8-2007

Venom Expenditure by Viperid and Elapid Snakes: Mechanisms, Adaptation, and Application

Shelton Scott Herbert

LOMA LINDA UNIVERSITY
School of Science and Technology
in conjunction with the
Faculty of Graduate Studies

Venom Expenditure by Viperid and Elapid Snakes: Mechanisms, Adaptation, and Application

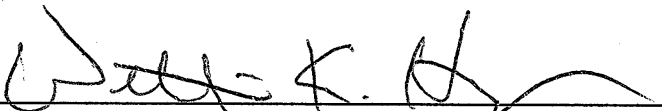
by

Shelton Scott Herbert

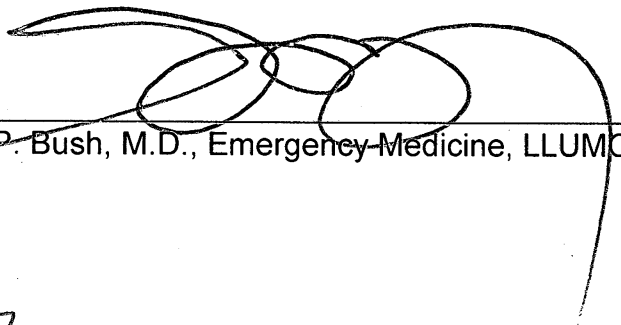
A Dissertation submitted in partial satisfaction of
the requirements for the degree of
Doctor of Philosophy in Biology

August 2007

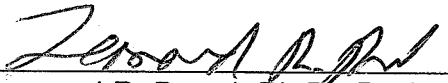
Each person whose signature appears below certifies that this thesis, in his opinion, is adequate in scope and quality as a dissertation for the degree Doctor of Philosophy.



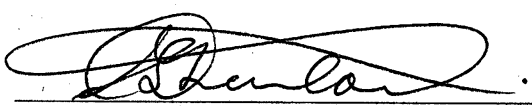
William K. Hayes, Ph.D., Professor of Biology, Chairperson



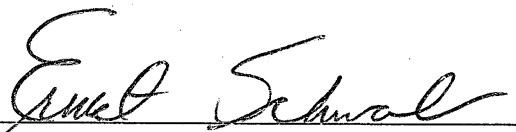
Sean P. Bush, M.D., Emergency Medicine, LLUMC



Leonard R. Brand, Ph.D., Professor of Biology and Paleontology



Stephen G. Dunbar, Ph.D., Assistant Professor of Biology



Ernest R. Schawb, Ph.D., Associate Professor of Allied Health Studies

ACKNOWLEDGMENTS

I owe much to many! However, there are those to whom I am especially indebted. My love of science I owe to my parents. Without their example and enthusiasm, I would not now know the enchantment that science holds for me. To my wife and daughter, I express a most profound gratitude for their patience and forbearance of my absence from home in the name of research.

I deem myself fortunate for all those I count as my extended family. This includes many who have served in the Department of Earth and Biological Sciences. To Dr. Brand, Dr. Carter, and Dr. Cushman as department chairs, who showed great patience and support, I thank you. I also wish to thank those whose support was perhaps more subtle, yet still immensely important–those who served as department secretaries: Judy Cushman, Patti Perrin, and Valerie Dekle. Without their help, I certainly would not have been able to reach this point! Thank you for your caring, patience, persistence, and outright ingenuity on my behalf!

It has been a blessing to share my academic journey with other students! People with whom I've performed research, bounced ideas off of, and have been a part of my life over the past few years. I especially wish to thank Jim Smith and Tony Trimm, who shared my early years at Loma Linda, and Zia Nisani, who has been most persistent and supremely effective with his unique brand of humor-enriched support and friendship. To have such friends is a great fortune.

Finally, I end with the person to whom I owe the deepest gratitude. Dr. William Hayes has been a source of profound inspiration, contagious

enthusiasm, and has been a most intuitive instructor. His knowledge, patience, and his humorous outlook combined to provide supremely effective instruction. He has invested tremendous amounts of time and energy on my behalf. While I am indeed grateful to have had Dr. William Hayes as my professor and mentor, I consider it my greatest fortune to call him my friend!

CONTENTS

TABLES

FIGURES

Figures Page

ABSTRACT OF THE DISSERTATION

Venom Expenditure By Viperid And Elapid Snakes:
Mechanisms, Adaptation, And Application

by

Shelton Scott Herbert

Doctorate of Philosophy, Graduate Program in Biology
Loma Linda University, August 2007
Dr. William K. Hayes, Chairperson

In this dissertation, I examined some of the factors that influence venom expenditure by viperid and elapid snakes in both predatory and defensive contexts. I also considered the consequences of venom delivery into human snakebite victims. In the first of four experiments, In the first experiment, I explored whether the Black-necked Spitting Cobra (*Naja nigricollis*) metered venom by differential venom gland contraction. Differences in duration of venom flow and venom expended confirmed that this species ejects markedly greater quantities of venom during biting than spitting by varying the duration of venom gland contraction. In the second experiment, I studied the effects of varying levels of perceived threat on how snakes bite defensively and allocate their venom. Two viperid snakes (*Calloselasma rhodostoma, Bothrops atrox*) and one elapid (*Naja annulifera*) demonstrated risk assessment by biting more quickly and expending more venom when biting model human limbs at higher levels of threat. In the third experiment, I examined whether rattlesnakes expend optimal quantities of venom when feeding on rodent prey. The results supported my prediction that the quantity of venom rattlesnakes typically inject into mice

produces the most rapid incapacitation and death for the least amount of venom. Moreover, the optimum dose for securing larger rodent prey should be greater than that for smaller prey, In the fourth experiment, I explored the potential of denim cloth (i.e., blue jeans) to interfere with and reduce the amount of venom injected during a defensive bite to a human. When Southern Pacific rattlesnakes (*Crotalus oreganus helleri*) were provoked to bite bare and denim-covered human limb models, the presence of denim reduced the amount of venom injected by approximately two-thirds for both small and large rattlesnakes. Thus, clothing can be considered a simple, low-cost, and potentially effective means of providing a measure of protection from snakebite when in the habitat of venomous snakes. Collectively, these studies add to a growing body of literature documenting the mechanisms, adaptive value, and human importance of venom expenditure by snakes.

CHAPTER I

INTRODUCTION TO VENOM EXPENDITURE BY SNAKES

In this dissertation, I examine some of the factors that influence venom expenditure by viperid and elapid snakes in both predatory and defensive contexts. I also consider the consequences of venom delivery into human snakebite victims. In this chapter, I begin by reviewing basic information about venomous snakes, the biological roles or functions of venom, and how these relate to the mass of venom expended during a bite. I then explore the mechanisms that regulate venom expenditure, how variation in venom expenditure can be adaptive, and why the study of venom expenditure should be applied to issues of human safety.

Venomous Snakes and their Weapons

Venomous snakes are recognized from four snake families: Viperidae, Colubridae, Atractaspididae, and Elapidae. Three of these groups possess front-fanged venom delivery systems, whereas the fourth, Colubridae, relies on a rear-fanged system (Underwood, 1967; Underwood and Kochva, 1993; Vidal, 2002). Among these families, the viperids and elapids pose the greatest risks to humans because of their diversity, widespread distribution, and capacity to delivery large doses of highly toxic venom.

The venom of front-fanged snakes is stored in paired venom glands surrounded by muscles which contract to expel the venom through the venom ducts to a pair of hollow-tipped fangs (Haas, 1973; Kardong and Lavin Murcio, 1993; Kochva, 1978; Mackessy, 1991; Rosenberg, 1967; Young et al., 2000, 2001a; Young and Zahn, 2001). Venom entering the tissues of a target causes damage ranging from discomfort and tissue injury to death. The venom apparatus and kinematics of biting are designed to deliver large quantities of venom during a relatively brief period of fang contact (e.g., Gans, 1961; Kardong, 1982; ; Kochva, 1987; Mackessy, 1991; Kardong and Lavin Murcio, 1993; Kardong et al., 1997; Kardong and Bels, 1998; Jackson, 2003; Young and Jackson, 2007; Fry et al., in press). In contrast to the front-fanged snakes, rear-fanged colubrids lack the sophisticated apparatus (large venom glands and storage reservoir, hollow teeth, venom gland musculature) for delivering large amounts of venom efficiently and quickly into the tissues of a target. The toxic secretions in the saliva must enter the target's tissues by seeping in as the snake chews.

The venom of these snakes represents a complex mixture of liquids and toxic proteins which the snakes produce and store within their paired venom glands (reviewed by Tu, 1977, 1982, 1991; Kochva, 1987; Chippaux et al., 1991; Thorpe, 1997; Aird, 2002; Gutierrez, 2002; Fry and Wuster, 2004; Fry, 2005; Fry et al., 2005, in press; Hodgson and Wickramaratna, 2006). Most viperid snakes possess venom that is primarily proteolytic, digesting tissues and causing considerable pain. However, the venom of some species possesses neurotoxic

properties which interfere with the electrochemical conduction of impulses to vital bodily functions, particularly those of the respiratory system (reviewed by Werman, in press). Most elapid snakes possess venom that is primarily neurotoxic, typically eliciting less pain upon injection but generally leading to more rapid death.

Biological Roles of Venom

The primary roles of snake venom are to procure food (predation) and to protect against attack (defense). In both cases, venom may meet several needs (Hayes et al., 2002). When acquiring food, venom serves to rapidly immobilize and kill the prey, facilitate relocation of prey, and accelerate digestion of prey. Most snakes swiftly strike, envenomate, and voluntarily release larger prey items, which minimizes the risk of sustaining retaliatory injury (Kardong, 1986a). Prey that are released often travel several meters or more before dying, making it necessary for the snake to relocate its victim (Kuhn et al., 1991; Hayes, 1992a). The venom alters the scent of the prey such that the snake is able to relocate its meal by following the odoriferous trail deposited by the envenomated animal (Chiszar et al., 1992; 1999; Lavin Murcio et al., 1993; Kardong and Smith, 2002). The proteolytic properties of venom also accelerate digestion, which may prevent putrefaction and regurgitation of larger, bulkier prey (Thomas and Pough, 1979; Rodriguez-Robles and Thomas, 1992); however, this view has been challenged recently (McCue, 2007). Depending on local prey availability or other factors, selection may act on venom components for any of these functions

independently of or in tandem with other functions (Aird, 2002; Chiszar et al., 1999).

When confronted by predators (e.g., canids, raptors) or antagonists (e.g., ground squirrels, ungulates, humans), snakes also rely on venom for defense. It is important to distinguish between predators (which attack the snake to consume it) and antagonists (which harass or attack the snake but have no intention of eating it), because the snake's strategy for survival may vary with context of the attack (Hayes et al., 2002). Snakes appear to benefit from defensive use of their venom in both proximate (current mechanisms) and ultimate (adaptations via natural selection) ways. Because a defensive bite is highly unlikely to cause death of the attacker before the snake itself dies, the proximate benefit to the snake is that a painful bite may often terminate an attack, allowing the snake to survive. In ultimate terms, the lethal bite confers protection against attack from predators that have been selected to avoid or reduce predation on snakes or to interact with them in a more cautious manner (e.g., Coss et al., 1993; O'Connell and Formanowicz, 1998; Owings and Coss, 2007). Given these considerations, the effectiveness of envenomation during defensive bites may vary with composition of venom or biochemical resistance of the target animal. Neurotoxic venoms, for example, do not elicit painful sensations as readily as hemorrhagic venoms (e.g., Minton, 1987). Thus, selection may favor particular venom components not only for their roles in procuring food but also for their effectiveness at defense.

Mechanisms Regulating Venom Expenditure

Recent debate has emerged on the capacity of snakes to control, or meter, how much venom is injected during a bite (reviewed by Hayes, 2007). Proponents of venom metering conclude that snakes have the cognitive (i.e., decision-making) capacity to control, or meter, how much venom is ejected from the fangs (Hayes et al., 1995, 2002). Experimental support derives from measures of venom expended by snakes while biting in different contexts (predatory versus defensive) and at different target properties (e.g., size). Proponents of the pressure-balance hypothesis, in contrast, attribute differences in venom expenditure to variation in strike kinematics and/or target surface features (Young et al., 2002, 2003; Young and Kardong, 2007; Young, 2007). Although these hypotheses represent different levels of analysis (cognitive and physiological mechanisms, respectively; Hayes, 2007) and are not mutually exclusive, they have frequently been pitted against each other as alternative explanations.

Proponents of the pressure-balance hypothesis have argued, largely from lack of evidence, that snakes are incapable of neural regulation of venom gland contraction (Young et al., 2002; Young, 2007). In their view, venom gland compression results in an invariably-sized bolus of venom that is propelled through the ducts to the fang sheath and then out of the hollow fangs. Ordinarily, the fang sheath membranes cover the fangs and internally block the entrance of venom into the fangs (Young and Kardong, 2007). During biting (or spitting by spitting cobras), the fang sheath becomes compressed (elevated toward the

base of the fangs), exposing the fangs and removing the internal soft tissue barrier to venom flow. The degree of fang sheath compression, which could be influenced by target features, might have an overriding influence on the passage of venom through the fangs. Thus, several pertinent questions arise. To what extent can a snake control the amount of venom expended via differential venom gland contraction? Does fang sheath compression have a greater influence on venom expenditure, and if so, can the snake still control this? A recent experimental study sought to compare the relative influence of venom gland contraction and fang sheath compression; however, the study design and conclusions were flawed (Young and Kardong, 2007).

In Chapter 2, I explore whether venom metering could occur by means of differential venom gland contraction. That is, can snakes control venom expulsion by varying the force and/or duration of venom gland contraction? By comparing the duration of venom flow during biting and spitting in the Black-necked Spitting Cobra (*Naja nigricollis*), I conclude that this species is able to eject markedly different quantities of venom during biting and spitting by varying the duration of venom gland contraction. Because of functional convergence in the design and regulation of the venom delivery system between viperids and elapids, I suggest that other venomous snakes likewise have the ability to regulate venom gland contraction.

Adaptive Features of Venom Expenditure

It may be advantageous for a snake to be judicious when deploying its venom. Venom can be viewed as a limited commodity due to the metabolic costs of replacing it and the ecological costs of a depleted supply of venom. Although the metabolic costs of venom synthesis may not be high, they exceed those for growth of normal body tissue and can represent a modest portion of caloric intake from a meal (McCue, 2006). A snake with insufficient venom may be unable to procure additional prey or defend itself against attack until its supply of venom has been at least partially restored (Hayes et al., 1995, 2002). The amount of time required to replenish venom is poorly understood. When the venom glands are completely emptied (e.g., by forceful venom extraction), up to two weeks may be required to refill the glands (Kochva, 1960; Leinz and Janeiro-Cinquini, 1994; Schaeffer et al., 1972). Presumably less time is required after expenditure of smaller venom quantities, but this hypothesis has not been tested.

In addition to the need for conserving a valuable commodity, the optimal amount of venom to expend may vary with context of use. Prey that are larger in size or more resistant to venom, for example, may be more effectively procured or digested when more venom is injected (Hayes et al., 2002). Smaller prey, such as neonatal rodents, are often captured and consumed without any apparent use of venom (Klauber, 1972; Radcliffe et al., 1980). The amount of venom used in a defensive bite may vary depending on the identity of the attacker or the level of perceived threat. A snake that is physically grasped by an attacker, for example, is likely to inject more venom because the immediate risk

of death is far greater than the risk of having depleted supplies subsequently (Hardy, 1991; Herbert, 1998).

In Chapter 3, I focus on the effects of varying levels of perceived threat on the characteristics of venom expended. Snakes of two viperid and one elapid species were encouraged to bite when presented with low threat (threat stimulus moved in proximity to the snake), medium threat (threat stimulus repeatedly brought into contact with snake's body), and high threat conditions (snake grasped by the neck and body and its mouth held in light contact against a membrane-covered beaker). The results support my view that snakes demonstrate risk assessment in both eliciting the strike and the amount of venom injected when biting. Several features of venom expulsion, in particular the duration of venom flow, also support my view that snakes vary the duration and force of venom gland contraction.

In Chapter 4, I examine whether rattlesnakes expend optimal quantities of venom when feeding on rodent prey. I asked, "what amount of venom provides the optimal tradeoff between the shortest time to immobilization or death of prey and the least amount of venom expended?" To evaluate this, I injected mice, rats, and hamsters with different quantities of venom and recorded their time to immobilization and death. The quantities found to be optimal correspond well with the amounts rattlesnakes are known to inject when feeding on mice. The results also support my prediction that the optimum dose for securing larger rodent prey (rats and hamsters) should be greater than that for smaller prey (mice), matching the pattern of venom expenditure documented in behavioral

studies of snakes. The results add to a growing body of evidence supporting adaptive use of venom by snakes.

Applied Aspects of Venom Expenditure

Depending on the type and quantity of venom injected, bites to humans can be potentially painful, injurious, and even life-threatening. Recent studies suggest that more than 1 million venomous snakebites occur globally each year, resulting in as many as 100,000 deaths and countless more cases of long term disability (e.g., Chippaux, 1998, 2006; Gutierrez et al., 2006). The personal and financial costs of venomous bites can be substantial. The costs can include, but are not limited to, transport to a hospital and often between hospitals, emergency room treatment and hospitalization, antivenom administration, surgical intervention, and subsequent physical and/or occupational therapy. Additional costs borne by the patient or family include lost income from time off work or death. Although mortality is relatively rare, particularly in developed countries, morbidity can exact an extraordinary toll (e.g., Dart et al., 1992; Spiller and Bosse, 2003; Gutierrez et al., 2006).

Clearly, any strategies that could reduce the amount of venom injected into a human target would likely reduce the severity of injury and costs associated with the bite. Studies of bite kinematics (Kardong, 1986b; Kardong and Bels, 1998; Young et al., 2001b) and their influence on venom expulsion (Hayes et al., 2002; Hayes, 2007) suggest a plausible link between studies of venom expenditure and snakebite risk to humans. Because venom delivery is

subject to disruption during a bite, protective clothing, even which fangs can penetrate, might suffice to reduce the amount of venom a snake injects.

In Chapter 5, I explore the potential of denim cloth, frequently worn as "blue jeans," to interfere with and reduce the amount of venom injected into model human limbs during defensive bites by rattlesnakes. I show that the presence of denim reduces the amount of venom injected by approximately two-thirds, and this was consistent for both small and large rattlesnakes. I also present evidence that the reduction results from the cloth interfering with venom delivery. I conclude that clothing can potentially result in a substantial reduction of venom injected and, thus, a notable reduction in the likely severity of the bite.

Chapter II

Venom Metering During Spitting Versus Biting: Differential Venom Gland Contraction Regulates Venom Expenditure in the Spitting Cobra, *Naja nigricollis*

William K. Hayes[1], Shelton S. Herbert[1], James R. Harrison[2], and Kristen L. Wiley[2]

[1]Department of Earth and Biological Sciences, Loma Linda University, Loma Linda, CA 92354 USA
[2]Kentucky Reptile Zoo, Slade, KY 40376 USA

This chapter has been accepted for publication with the following citation:

Hayes, W. K., S. S. Herbert, J. R. Harrison, and K. L. Wiley. In press. Venom metering during spitting versus biting: differential venom gland contraction regulates venom expenditure in the spitting cobra, *Naja nigricollis*. Journal of Herpetology.

ABSTRACT

According to the venom-metering hypothesis, snakes have the cognitive capacity to control, or meter, how much venom is ejected from the fangs. Critics of venom metering have argued, largely from absence of evidence, that differential venom gland contraction in snakes is trivial or nonexistent. To address this criticism, we videotaped the defensive bites of *Naja nigricollis nigricollis* during routine venom extractions. Mean duration of venom flow during a single pulse from a fang during biting (0.35 sec) was significantly longer than that reported previously for spitting (0.066 sec). Moreover, mean mass of venom expended per pulse from a fang during biting (juveniles: 14.2 mg; adults: 188 mg) significantly exceeded that reported for spitting (1.85 mg). During a single bite,

both juveniles and adults delivered venom via pulses that were single, multiple (each associated with a jaw contraction), unilateral (from one fang), and/or bilateral (from both fangs more or less simultaneously). Although juveniles and adults exhibited similar venom flow duration, adults delivered ten-fold more venom during biting at four-fold greater rates of venom flow through the fang. Because venom gland contraction provided the only propulsive force for the venom expulsion, our results confirm that *N. n. nigricollis* meters larger quantities of venom during biting than spitting via differential venom gland contraction. Because of the high degree of functional convergence between venom delivery systems of elapids (including spitting cobras) and viperids, the capacity for differential venom gland contraction may be widespread among snakes.

INTRODUCTION

Two major hypotheses have been proposed to explain variation in venom expenditure by snakes. The venom-metering hypothesis, supported by experiments evaluating different contexts (predatory versus defensive) and target properties (e.g., size), proposes that snakes have the cognitive (i.e., decision-making) capacity to control, or meter, how much venom is ejected from the fangs (reviewed by Hayes et al., 2002; Hayes, 2007). The pressure-balance hypothesis, in contrast, attributes differences in venom expenditure to variation in strike kinematics and/or target surface features (Young et al., 2002, 2003; Young and Kardong, 2007; Young, 2007). Although these hypotheses represent different levels of analysis (cognitive and physiological mechanisms, respectively;

Hayes, 2007) and are not mutually exclusive, they have frequently been pitted against each other as alternative explanations.

Proponents of the pressure-balance hypothesis have argued, largely from lack of evidence, that snakes are incapable of neural regulation of venom gland contraction (Young et al., 2002; Young, 2007). In their view, venom gland compression results in an invariably-sized bolus of venom that is propelled through the ducts to the fang sheath and then out of the hollow fangs. Ordinarily, the fang sheath membranes cover the fangs and internally block the entrance of venom into the fangs (Young and Kardong, 2007). During biting (or spitting), the fang sheath becomes compressed (elevated toward the base of the fangs), exposing the fangs and removing the internal soft tissue barrier to venom flow. Based on experimental analysis of the Western Diamondback Rattlesnake (*Crotalus atrox*), Young and Kardong (2007) concluded that the relative influence of muscle contraction on venom flow was roughly $1/10^{th}$ that of the fang sheath. However, their experimental methods cast doubt on the validity of their conclusion (Hayes, 2007; see also the Discussion below).

Spitting cobras (members of the genus *Naja*) present an excellent model for examining the role of differential venom gland contraction in venom expulsion. Spitting cobras deploy their venom by both biting and spitting. Spitting occurs in a defensive context, and involves a brief stream of venom expelled from the fangs that is generally aimed at the eyes of a predator or antagonist (Young et al., 2004; Westhoff et al., 2005). Because individual spits are very brief in duration (ca. 0.06 sec in *Naja nigricollis* and *N. pallida*; Young et al., 2004; Westhoff et al.,

2005) and involve only small quantities of venom (ca. 4 mg in *N. nigricollis* and *N. pallida*; Freyvogel and Honegger, 1965; Cascardi et al., 1999), spitting cobras can often generate 40 or more spits before exhausting their venom supply (Cascardi et al., 1999; Westhoff et al., 2005). In contrast to spitting, these same species routinely yield substantially more venom during defensive bites (e.g., 300 mg or more during venom extractions of *N. nigricollis*; Sprawls and Branch, 1995; J. R. Harrison and K. L. Wiley, pers. obs.).

These observations raise intriguing questions. How are spitting cobras able to deliver more venom during a bite compared to a spit? Does the snake give many rapid, small pulses of venom during a defensive bite, similar in volume to spitting, or does it simply give one or several exceptionally large pulses of venom? The latter explanation would allow one to infer that the differences in venom expenditure between biting and spitting result from differential contraction of the venom gland, as proposed earlier by Hayes et al. (2002).

The primary objective of this study was to determine the duration of venom flow from the fangs of a representative spitting cobra, *N. nigricollis nigricollis*, during a defensive bite, and to compare this duration to that previously reported for spitting. From these values, we could infer whether differences in venom expenditure between biting and spitting result from differential venom gland contraction. By using both juvenile and adult snakes, and by measuring the quantities of venom ejected during the bites, we could also evaluate relationships among venom flow duration, venom expenditure, and ontogeny.

METHODS

Subjects.—Six long-term captive adult (5 ♂♂, 1 ♀; 152-165 cm snouth-vent length, SVL) and four 1-yr-old captive-born juvenile (3 ♂♂, 1 ♀; 99-107 cm SVL) *N. n. nigricollis* were kept in the Kentucky Reptile Zoo facility for routine (2/month) venom extractions. The adults were housed in 61 x 61 x 46 cm (L x W x H) plastic cages and the juveniles in 61 x 46 x 15 cm (L x W x H) plastic cages with aspen or cypress shavings as substrate. The snakes were maintained at 26-30°C on a 12:12 L:D photoperiod. The snakes were provided water *ad libidum* in a small ceramic vessel and fed pre-killed rodents on a regular basis (rats twice per month to adults, mice once per month to juveniles).

Venom extractions.—Venom flow during biting was videotaped (Sony model DCR- TRV300, 8 mm digital, 30 fields/sec) twice, 10 mo apart, during regularly-scheduled venom extractions. The first extraction included only adults; the second included both adults and juveniles. Each snake was pinned by hook, grasped behind the head, and the mouth pushed gently against the rim of 10 cm wide glass funnel covered with a Parafilm membrane. For consistency and safety, a single individual (JRH) conducted all venom extractions. Bites by the snakes were voluntary (Glenn and Straight, 1982). No external pressure was applied to the glands; thus, venom propulsion from the fangs was presumably generated solely by venom gland contraction (Young et al., 2002; Young, 2007). The camera was positioned ca. 30-40 cm from the funnel at ca. 10-20° below the horizontal plane of the funnel membrane. The camera view was rostral to (directly in front of) the snake's snout, allowing simultaneous views of both fangs.

Individual venom samples collected from the second extraction were frozen, lyophilized using a Labconco manifold freeze-dryer and a Leybold D4A pump, and weighed to the nearest 10 mg.

In the first session, four of the adults (snakes A, B, D, E) expended venom and provided satisfactory videos. In the second session, all six adults delivered venom, but video images were sufficiently clear for only three (snakes B, D, and E). All four juveniles also expelled venom, but video images were clear for only three (snakes G, I, J). Thus, videos of 10 bites were examined. Because the bite characteristics of snakes tested twice differed considerably, we chose to include all available data and treated all extractions as independent.

Video review.—Videotapes were reviewed frame-by-frame to quantify variables associated with venom flow. Resolution was a single video field (0.033 sec); however, for convenience, we report all durations here to the nearest 0.01 sec. A venom pulse was a discrete episode of continuous venom flow (ejection) from a single fang, measured as the number of fields during which flow was visible. Multiple pulses involved two or more such episodes separated by an interval of no venom flow. We recorded the duration of each successive pulse and the interval between them. Venom pulses were associated with unambiguous jaw contractions that were counted. These jaw movements involved a forceful downward thrust of the upper jaw (somewhat below the plane of the funnel membrane) as a pulse commenced, followed by rotation of the upper jaw upward (toward the plane of the funnel membrane) after a pulse terminated. Vertical motion was more subtle between multiple contractions

compared to the initial and final jaw movements. The mass of venom expended was divided by the duration of venom flow (summed for all pulses) to derive the rate of venom flow (mg/sec) per pulse per fang. Because flow rates were variable during each extraction, typically beginning with a steady stream but then trailing off to a trickle, these values were regarded as the average rate of venom flow during the entire episode of venom expulsion. The mass of venom expended was also divided by the total number of pulses (summed for both fangs) to calculate venom expended per pulse per fang. We considered all extractions to consist of a single bite, though one had sufficient lapse between successive pulses to be interpreted as two bites.

Analyses.—Most data failed to meet parametric assumptions and, accordingly, were subjected to nonparametric tests (Conover, 1999). We usually treated independent and dependent data separately; however, in several instances (as specified in Results), we pooled independent and related data, assuming all to be independent. Of the two one-sample *t*-tests used, one involved a mild problem with normality (see Results). For the Analysis of Covariance (ANCOVA) involving a very small data set (precluding tests of assumptions), we sought only to compute effect sizes for comparing the influence of age class (snake size) and total pulse duration on venom expenditure. All tests were two-tailed, unless otherwise specified, with alpha set at 0.05.

RESULTS

Patterns of venom pulses.—Venom expulsion varied remarkably among the 10 venom extractions (Table 2-1). Five (50%) of the venom extractions involved just one pulse of venom ejected from one or both fangs, three (30%) involved two pulses from at least one fang, and two (20%) involved three pulses from a single fang. Each of the successive pulses was associated with a distinct jaw contraction (Table 2-1). Individual pulses were either bilateral (venom ejected from both fangs) or unilateral (venom ejected from one fang). Four (40%) of the 10 first pulses, four (80%) of the five second pulses, and both (100%) of the third pulses were unilateral (Table 2-1). Multiple, unilateral, and bilateral pulses were exhibited by both juveniles and adults (Table 2-1).

Pulse durations.—We found no evidence for side dominance in venom expulsion. The mean ($\pm$ 1 S.E.) pulse duration was similar for the first pulse for left (0.39 $\pm$ 0.13; n = 6) and right (0.25 $\pm$ 0.05; n = 10) fangs, though analysis was limited to the matched pairs (Wilcoxon exact $P = 0.88$; n = 6). Two of the ten venom extractions involved multiple pulses by the left fang and four involved multiple pulses by the right fang (Table 2-1). When all pulses were assumed to be independent (i.e., pooling independent and related data), the total number of pulses by the left (9) and right (15) fangs was similar (Binomial exact $P = 0.31$).

We also found no evidence that venom expulsion declined between the first and second pulses (c.f., Hayes et al., 2002). When pulses were summed for both fangs and divided by two to represent mean duration of venom flow per fang, the pulse durations were similar for the first (0.24 $\pm$ 0.06 sec; $N = 10$) and

second (0.33 ± 0.13 sec; $N = 5$) pulses, though analysis was limited to the matched pairs (Wilcoxon exact $P = 0.81$; $N = 5$). Because three of the first pulses were bilateral and only one of the second pulses was bilateral (Table 2-1), we repeated this test using only the one fang that ejected venom in both pulses (Table 2-1) and reached the same conclusion (Wilcoxon exact $P = 0.25$; $N = 5$). Although the third pulse seemed comparatively brief (0.10 ± 0.02 sec; $N = 2$), only two extractions involved a third pulse and in neither case was it the pulse of briefest duration (Table 2-1). The duration of venom flow during successive pulses from the same fang was consistent in one extraction (0.17 sec in all three pulses from the left fang of snake II-J-juv) but varied by more than four-fold in two others (snakes I-A-ad and II-B-ad; see Table 2-1).

Pulse durations appeared to be similar for juvenile and adult snakes. When the pulse 1 durations from the right fangs were compared (Table 2-1), there was no difference between juveniles (0.23 ± 0.04 sec; $N = 3$) and adults (0.26 ± 0.07 sec; $N = 7$; Mann-Whitney exact $P = 1.00$). When the total pulse durations were compared (i.e., summed for both right and left fangs and for all three pulses), there was, again, no difference between juveniles (0.74 ± 0.32 sec; $N = 3$) and adults (0.90 ± 0.23 sec; $N = 7$; Mann-Whitney exact $P = 0.83$).

The most important analysis was whether venom pulse duration during biting, measured here, exceeded that reported in a previous study for spitting (mean = 0.066 sec from one specimen of unspecified size between 45-130 cm SVL, Young et al., 2004; 0.048-0.060 from another specimen between 150-180 cm SVL, Westhoff et al., 2005). Because the bite analyses above suggested

independence between pulses from right and left fangs and among the three successive pulses, we pooled all of the pulses (including both independent and related data, mildly failing a Kolmogorov-Smirnov test for normality: $P = 0.049$) for comparison to the single value for spitting. Indeed, a one-sample *t*-test confirmed that the mean pulse duration per fang during biting (0.35 $\pm$ 0.07 sec; 95% CI = 0.15-0.43 sec; $N = 24$) differed significantly from the 0.066 sec value for spitting ($t = 4.21$, df = 23, $P < 0.001$). After removing the two pulses exceeding 1.0 sec duration (resulting mean = 0.27 + 0.03 sec; 95% CI = 0.20-0.34), another one-sample *t*-test yielded the same level of significance ($t = 5.98$, df = 21, $P < 0.001$). Because of the positive skew, the median value (for all 24 pulses) of 0.25 sec might better represent the duration of venom gland contraction during biting, which was nearly four-fold greater than that of spitting. Pulse duration varied from 0.07 - 1.55 sec. Only one pulse (0.07 sec of extraction I-B-ad) of the 24 pulses measured was similar in duration to the 0.066 sec reported duration of spits by *N. nigricollis*.

Intervals between pulses.—The interval between successive pulses was usually brief (0.13-0.30; $N = 6$, pooling both first and second intervals in Table 2-1). However, this range excluded the one extreme value of 2.97 sec between successive right-fang pulses of extraction II-E-ad (Table 2-1), an extraction that could be interpreted as two bites. Although not compared statistically, the intervals seemed similar between the first and second and between the second and third pulses, and were comparable to durations of venom pulses.

Venom expended.—A number of studies confirm that larger snakes expend more venom than smaller snakes (reviewed by Hayes et al., 2002; Hayes, in 2007). Also, larger snakes have proportionately larger fang lumens (Klauber, 1936) that can accommodate greater venom flow during venom expulsion (Herbert, 1998). Thus, one-tailed hypotheses were used to compare venom expenditure, venom flow rate, and venom per pulse between juvenile and adult snakes. As expected, adults (365 $\pm$ 161 mg; N = 6) expended significantly (>10-fold) more venom than juveniles (30 $\pm$ 14 mg; N = 4; Mann-Whitney one-tailed exact P = 0.034; Table 2-1). Venom flow rate was also greater (four-fold) for adults (233 $\pm$ 124 mg/sec; N = 3) than juveniles (58 $\pm$ 19 mg/sec; N = 3; Mann-Whitney one-tailed exact P = 0.05). However, although the amount of venom per pulse was more than eight-fold greater for adults (188 $\pm$ 65 mg/pulse; N = 3) than juveniles (14.2 $\pm$ 2.2 mg/pulse; N = 3), the difference was not significant (Mann-Whitney one-tailed exact P = 0.20).

Because of substantial skew, we used a binomial test rather than a one-sample *t*-test to determine whether venom expenditure during biting by all snakes in our study (pooling adults and juveniles) exceeded that reported in a previous study for spitting (mean = 3.7 mg per spit or 1.85 mg per fang; Freyvogel and Honegger, 1965). The mean venom expended per pulse was 66 $\pm$ 37 mg (95% CI = -29-161; N = 6), with values ranging from 10-233 mg. The probability that all six values for bites exceeded the 1.85 mg value for spits was significant (Binomial exact P = 0.031), confirming greater venom expenditure during biting compared to spitting.

Unfortunately, because adults and juveniles could not be pooled, the sample size was too small to detect a significant relationship between total duration of venom flow and quantity of venom expended (N = 3 for each age class). However, the relationship was clearly positive (Spearman's r_s^2 = 0.25 for each age class; Fig. 1). To compare effect sizes for age class and total pulse duration, a one-way ANCOVA model was employed, treating venom expended as a dependent variable, age class as a between-subjects independent variable, and total pulse duration as a covariate. The partial η^2 values for age class (0.23) and total pulse duration (0.25) were similar, suggesting that total pulse duration, like age class, exerted a significant influence on venom expenditure.

DISCUSSION

Critics of the venom-metering hypothesis—that snakes can control and make decisions about how much venom they expend—have argued against the possibility that snakes are capable of differential venom gland contraction (Young et al., 2002; Young, in press). Our primary purpose in this study was to evaluate whether a representative spitting cobra, *N. n. nigricollis*, expends different quantities of venom during spitting and biting by means of differential venom gland contraction. The results were unequivocal. The duration of venom pulses ejected from a fang during biting (mean = 0.35 sec; median = 0.25 sec) exceeded by nearly four-fold ($P < 0.001$) that reported for spitting (mean = 0.066 sec) in an earlier study (Young et al., 2004; c.f. Westhoff et al., 2005). The greater duration of venom flow corresponded to an exceptional dose of venom

ejected (mean = 188 mg/pulse and 365 mg total for adults, 14.2 mg/pulse and 30 mg total for juveniles), far more ($P = 0.031$) than that documented for spitting (mean = 1.85 mg/pulse) in an earlier study (Freyvogel and Honegger, 1965; c.f. Cascardi et al., 1999). In contrast to the consistency among spits within and between snakes (Freyvogel and Honegger, 1965; Cascardi et al., 1999; Young et al., 2004; Westhoff et al., 2005), bites involved pulse durations that varied remarkably, ranging from 0.07 - 1.55 sec.

Because venom gland contraction provided the only propulsive force for the venom expulsion (Young et al., 2002; Young, 2007), our results confirm that *N. n. nigricollis* meters different quantities of venom during spitting and biting by means of differential venom gland contraction. Spits involve very brief contraction, whereas bites almost always involve lengthy contraction. Although not considered here, differences in the force of venom gland contraction are also possible.

We did not expect, nor did we see, differences between the left and right fangs (side dominance). Had differences occurred, they might have been attributed to asymmetrical presentation of snakes to the venom collection apparatus (all snakes were grasped by JRH's right hand). We also failed to find differences between consecutive pulses. Other studies demonstrate that venom expenditure varies considerably between consecutive bites, but a decline—presumably arising from depletion of venom reserves—may not happen until after the first few bites (Hayes et al., 2002; Hayes, 2007).

The pulse durations and venom expenditure showed contrasting patterns of ontogeny. The venom pulses associated with biting were similar for both age groups. Multiple, unilateral, and bilateral pulses were observed in both age classes, and the duration of pulses was similar. The quantity of venom injected, however, was much greater for adults ($P = 0.034$), as expected by virtue of their greater supply of venom (Hayes et al., 2002; Hayes, 2007). Likewise, venom flowed through the fangs at rates much greater for adults than juveniles ($P = 0.05$), as documented previously for rattlesnakes (*Crotalus oreganus* ssp.) and cottonmouths (*Agkistrodon piscivorus*) and attributed to the larger lumen of adult fangs (Herbert, 1998). Although the quantity of venom per pulse was statistically similar, values for adults exceeded juveniles by more than eight-fold and our test suffered from small sample size and lack of statistical power.

In spite of the small sample size, our data supported the view that the duration of venom flow corresponds to the quantity of venom expended. Based on effect sizes obtained from the ANCOVA model, this relationship (partial $\eta^2 = 0.25$) may be as strong as that between snake size and venom expenditure (partial $\eta^2 = 0.23$), which is well documented in a number of snake species (Hayes et al., 2002; Hayes, 2007). Similar data obtained from venom extractions of a large sample of rattlesnakes and cottonmouths provide stronger support for the positive relationship between duration of venom flow and quantity of venom expended (Herbert, 1998).

Venom delivery by cobras during defensive bites showed a number of similarities to other snakes. Both elapids (spitting cobras in this study) and

viperids (rattlesnakes and cottonmouths; Herbert, 1998; Hayes, 2007) exhibit independent control of each fang. Both are capable of delivering bilateral (from both fangs) or unilateral (from one fang) venom pulses during a bite. Both can deliver multiple venom pulses of independently-varying duration with a brief interval between successive pulses. In both groups, individual pulses are associated with jaw contractions that, in viperids, also involve fang retraction. Thus, venom delivery characteristics are similar for the two snake families despite well-documented differences in their venom delivery systems (Jackson, 2003; Young and Kardong, 2007) and prey capture behavior (Kardong et al., 1997).

Spitting cobras may be unique, however, in their ability to compress (or elevate) their fang sheath independent of contact with a target surface. This capacity, described by Young et al. (2004), is essential for spitting. Contraction of the M. protractor pterygoideus muscle(mean duration = 0.143 sec) causes displacement and deformation of the palato-maxillary arch and fang sheath, thereby removing soft tissue barriers within the fang sheath and permitting venom to flow. Subsequent contraction of the M. adductor mandibulae externus superficialis (mean delay after PP activation = 0.037 sec; mean duration = 0.096 sec) increases venom pressure within the venom gland, propelling venom through the venom duct and out the fang. Thus, spitting results from precise coordination between these muscle groups. Rattlesnakes and other viperids, in contrast, lack muscular control of the venom sheath, which is compressed passively during contact with a target as the fangs penetrate the target (Young

and Kardong, 2007). Even so, in both taxa, venom expulsion results from an additive effect of gland compression and fang sheath deformation (Young and Kardong, 2007). Accordingly, Young and Kardong (2007) concluded that there was a high degree of functional convergence within this system.

Given the high degree of functional convergence, we see no *a priori* reason why viperids, like spitting cobras, could not similarly control duration (or possibly force) of venom gland contraction. Indeed, our analyses of venom flow duration support this view (Herbert, 1998; Hayes, 2007). Recently, Young and Kardong (2007) evaluated this possibility experimentally in the Western Diamondback, *C. atrox*. They reported that fang sheath compression resulted in 10-fold greater increases in venom flow (peak pressure) compared to differential contraction of the compressor glandulae muscles acting on the venom gland. However, their experimental design raises questions concerning relevance. First, venom pressures during differential contraction of the gland muscles were explored without fang sheath compression; thus, the relationship interpreted as small (explaining 25-34% of variation in venom flow, which actually represents a large effect; Cohen, 1992) has little bearing on what takes place during a normal bite, when the fang sheath is necessarily compressed to allow fang penetration of the target. Second, no data were presented to show that differential fang sheath compression resulted in a stronger association with venom flow than that demonstrated for gland compression. Clearly, compression of the fang sheath is important to remove an internal block to venom flow, but does the difference between 80% and 100% compression actually affect venom flow? Finally, the

measure of venom flow in the study—pressure at the fang exit—simply does not correspond to quantity of venom ejected. The investigators did not evaluate or comment on venom flow duration, an arguably more important determinant of total venom expenditure.

Although control of venom gland contraction may be important, venom metering can occur through other mechanisms (Hayes, 2007). During the bite, for example, they can control how long the fangs remain in contact with the target and, therefore, how many pulses of venom are delivered. They can also deliver more than one bite. Additionally, they have some control of the residual momentum of the head upon contact with the target, the force and duration of jaw closure, the angle of fang erection (for viperids), and the depth of fang penetration (via jaw closure), all of which can influence pressures on the fang sheath.

In summary, we provide compelling data that demonstrate the ability of spitting cobras to meter different quantities of venom during spitting and biting through differential venom gland contraction. We see no reason why a similar mechanism would not exist in other snake taxa, which would provide an effective (though not essential) means for snakes to cognitively meter their venom.

LITERATURE CITED

Cascardi, J., F. A. Young, H. D. Husic, and J. Sherma. 1999. Protein variation in the venom spat by the red spitting cobra, *Naja pallida* (Reptilia: Serpentes). Toxicon 37:1271-1279.

Cohen, J. 1992. A power primer. Psychological Bulletin 112:155-159.

Conover, W. J. 1999. Practical Nonparametric Statistics (3rd ed). John Wiley & Sons, Inc., New York.

Freyvogel, T., and C. Honegger. 1965. Der 'Speiakt' von *Naja nigicollis*. Acta Tropica 22:289-302.

Glenn, J. L., and R. C. Straight. 1982. The rattlesnakes and their venom yield and lethal toxicity. Pp. 3-19 *in* A. T. Tu (ed.), Rattlesnake Venoms: Their Actions and Treatment. Marcel Dekker, New York.

Hayes, W. K. 2007. The snake venom-metering controversy: levels of analysis, assumptions, and evidence. In press *in* W. K. Hayes, K. R. Beaman, M. D. Cardwell, and S. P. Bush (eds.), The Biology of Rattlesnakes. Loma Linda University Press, Loma Linda, California.

Hayes, W. K., S. S. Herbert, G. C. Rehling, and J. Gennaro. 2002. Factors that influence venom expenditure in viperids and other snake species during predatory and defensive contexts. Pp. 207-233 *in* G.W. Schuett, M. Hoggren, M. E. Douglas, and H.W. Greene (eds.), Biology of the Vipers. Eagle Mountain Publ., Eagle Mountain, Utah.

Herbert, S. S. 1998. Factors influencing venom expenditure during defensive bites by cottonmouths (*Agkistrodon piscivorus*) and rattlesnakes (*Crotalus viridis, Crotalus atrox*). M.S. Thesis, Loma Linda University, Loma Linda, California.

Jackson, K. 2003. The evolution of venom-delivery systems in snakes. Zool. J. Linn. Soc. 137:337–354.

Kardong, K. V., T. Kiene, and E. K. Johnson. 1997. Proximate factors affecting the predatory behavior of the red spitting cobra, *Naja mossambic pallida*. J. Herpetol. 31:66-71.

Spawls, S., and B. Branch. 1995. The Dangerous Snakes of Africa. Ralph Curtis Books, Sanibel Island, Florida.

Westhoff, G., K. Tzschätzsch, and H. Bleckmann. 2005. The spitting behavior of two species of spitting cobras. J. Comp. Physiol. A 191:873-881.

Young, B. A. 2007. Perspectives on the regulation of venom expulsion in snakes. In press *in* W. K. Hayes, K. R. Beaman, M. D. Cardwell, and S. P. Bush (eds), The Biology of Rattlesnakes. Loma Linda University Press, Loma Linda, California.

Young, B. A., and K. V. Kardong. 2007. Mechanical and physiological influences on venom expulsion in the western diamondback rattlesnake, *Crotalus atrox*. J. Exp. Zool. 307A:18-27.

Young, B. A., C. E. Lee, and K. M. Daley. 2002. Do snakes meter venom? Bioscience 52:1121-1126.

Young, B. A., M. Phelan, M. Morain, M. Ommundsen, and R. Kurt. 2003. Venom injection in rattlesnakes (*Crotalus*): peripheral resistance and the pressure-balance hypothesis. Can. J. Zool. 81:313-320.

Young, B. A., K. Dunlap, K. Koenig, and M. Singer. 2004. The buccal buckle: the functional morphology of venom spitting in cobras. J. Exp. Biol. 207:3483-3494.

Table 2-1. Duration (sec) of venom pulses and interval between successive pulses in single-bite venom extractions of *Naja nigricollis nigricollis*. Roman numerals indicate the two venom extractions separated by 10 months and letters identify individual specimens; adult = ad; juvenile = juv.

Snake-Extraction	Pulse 1		Interval 1		Pulse 2		Interval 2		Pulse 3		Jaw Contractions
	L	R	L	R	L	R	L	R	L	R	
I-A-ad	-	0.13	-	0.30	0.20	0.56	-	-	-	-	2
I-B-ad	0.30	0.07	-	-	-	-	-	-	-	-	1
I-D-ad	0.26	0.23	-	0.20	-	0.10	-	0.13	-	0.23	3
I-E-ad	0.30	0.46	-	-	-	-	-	-	-	-	1
II-B-ad	-	0.33	-	0.30	-	1.55	-	-	-	-	2
II-D-ad	-	0.10	-	-	-	-	-	-	-	-	1
II-E-ad	0.26	0.50	-	2.97	-	0.69	-	-	-	-	2
II-G-juv	1.02	0.23	-	-	-	-	-	-	-	-	1
II-I-juv	-	0.17	-	-	-	-	-	-	-	-	1
II-J-juv	0.17	0.30	0.13	-	0.17	-	0.20	-	0.17	-	3
Mean (1 SE)	0.39 (0.13)	0.25 (0.05)	0.13	0.94 (0.68)	0.18 (0.02)	0.73 (0.30)	0.20	0.13	0.17	0.23	1.7 (0.3)

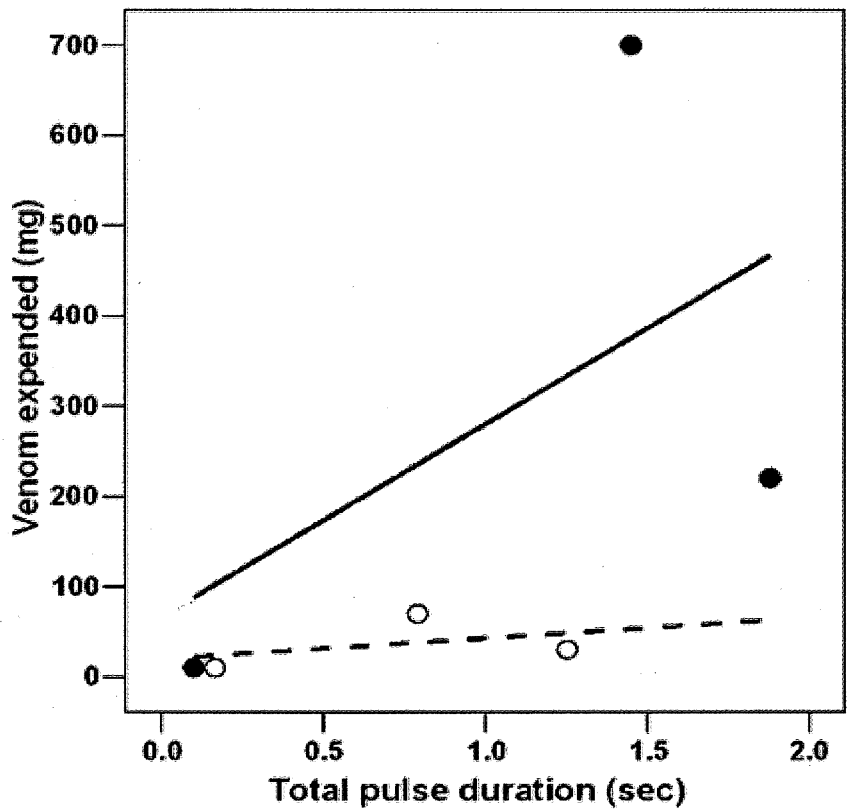


Figure 2-1. Relationship between total pulse duration (summed for all pulses from both fangs) and venom expended during venom extractions of juvenile (open circles, *N* = 3) and adult (solid circles, *N* = 3) spitting cobras, *Naja nigricollis nigricollis*.

Chapter III

Risk Assessment Influences Venom Expenditure During Defensive Bites by Viperid and Elapid Snakes

Shelton S. Herbert[1], William K. Hayes[1], James R. Harrison[2], and Kristen L. Wiley[2]

[1]Department of Earth and Biological Sciences, Loma Linda University, Loma Linda, CA 92354 USA
[2]Kentucky Reptile Zoo, Slade, KY 40376 USA

ABSTRACT

The degree of perceived threat has the potential to affect an animal's defensive behavior. In this study, we tested the responses of adults of three snake species (two vipers and one elapid) to three different levels of threat intensity. The vipers included *Calloselasma rhodostoma* and *Bothrops atrox*, and the elapid was *Naja annulifera*. Two threat levels involved a warm, saline-filled glove thrust toward the snake while either avoiding contact (low threat) or making repeated contact (medium threat) with the snake. In the high-threat condition, snakes were physically pinned and grasped by the investigator and allowed to bite a membrane-covered beaker. Each snake received a random sequence of threat presentations. The snakes were more likely to bite and bit more quickly at higher threat intensity. The snakes also expended more venom at higher threat intensity. Differences in venom expenditure appeared to be related to duration of venom flow through the fangs, suggesting the capacity of snakes to meter venom through differential venom gland contraction. During a single bite, all three species delivered venom via pulses that were single, multiple (each associated

with a jaw contraction), unilateral (from one fang), and/or bilateral (from both fangs more or less simultaneously). Among the species differences, *N. annulifera* was more reticent to bite, but when doing so, it maintained longer fang contact and venom flow, and likely delivered more venom. Collectively, the results suggest that venomous snakes readily assess the risk of threats and make decisions about whether to use their venom when biting and how much venom to inject.

INTRODUCTION

Many animals are capable of predator risk (or threat) assessment, allowing them to choose an appropriate response once the nature of a specific threat is identified. Most studies examining predator risk assessment have focused on choices involving conspicuous activities, such as foraging, courtship and mating, vigilance, fleeing or hiding, sleep, and defense of self or young (reviewed by Lima and Dill, 1990; Caro, 2005; Lima et al., 2005). Although risk assessment has been studied most frequently in vertebrates, even invertebrates demonstrate behavioral responses that vary with different levels of threat (e.g., Taylor et al., 2005).

Most venomous snakes readily use their venom to defend themselves. Because venom is a limited commodity, they presumably make decisions about whether or not to use their venom and how much to deploy when defending themselves (Herbert, 1998; Rehling, 2002; Hayes et al., 2002; Hayes, 2007). When a potential threat appears, the snake may attempt to escape, hide, bluff

(make threatening gestures of its own), or bite the animal posing the threat (Duvall et al., 1985; Goode and Duvall, 1989; Graves, 1989; Whitaker and Shine, 1999; Whitaker et al., 2000; Gibbons and Dorcas, 2002; Shine et al., 2002a; Shipman, 2002).

Should the snake choose to use its venom, it is capable of controlling, or metering, how much venom is expended (reviewed by Hayes et al., 2002; Rehling, 2002; Hayes, 2007). Although the majority of studies demonstrating venom metering examined venom expenditure during predatory bites, several studies suggest that venom metering also occurs with different levels of threat. When physically restrained during venom extractions (i.e., grasped by the head by a human), Cottonmouths (*Agkistrodon piscivorus*) and Cobras (*Naja kaouthia*) inject more venom than during unrestrained strikes at model human limbs (Herbert, 1998; Hayes et al., 2002). Southern Pacific Rattlesnakes (*Crotalus oreganus helleri*), in contrast, expend similar quantities of venom in the two contexts (Rehling, 2002).

The question of how a snake might deliver variable quantities of venom when biting has become a matter of recent debate (Hayes, 2007). Although a number of mechanisms exist by which a snake could control venom delivery (Hayes, 2007), the importance of one primary mechanism, differential venom gland contraction, has come into recent question (Young, 2007; Young and Kardong, 2007). Because venom expulsion is a product of the force and duration of venom flow, analyses of venom flow duration offer valuable insights on the potential for snakes to regulate venom gland contraction (Hayes, 2007). Indeed,

Black-necked Spitting Cobras (*Naja nigricollis*) rely on brief gland contractions to expel small volumes of venom when spitting and lengthy gland contractions to deliver larger volumes when biting (Hayes et al., in press). Comparative studies are needed to evaluate how widespread the capacity might be for differential venom gland contraction.

Risk assessment by an animal can be inferred from different behavioral choices made under varying threat conditions. The purpose of this study was to evaluate the effect of threat intensity on the defensive responses of three different snake species provoked to bite during three levels of escalating threat. The threat conditions were selected to represent threat levels likely to be encountered by the snake. We hypothesized that, with increasing threat, snakes would bite more readily and quickly, delivering greater amounts of venom. By quantifying venom expulsion during the high-threat condition, we also evaluated relationships between flow duration, rate of flow, and total venom ejected to infer properties of and constraints on venom gland contraction.

METHODS

Snakes.–Adults of three species representing two families (viperidae, elapidae) were used in a repeated-measures, multiple-threat study. The pitvipers included the Malayan Pitviper (*Calloselasma rhodostoma*; n = 10, snout-vent length (SVL) = 55-91 cm) and the Common Lancehead Viper (*Bothrops atrox*; n = 10, SVL = 95-104 cm). The Egyptian Cobra (*Naja annulifera*; n = 10, SVL = 109-170 cm) was the elapid. All snakes were kept in individual cages (61 x 61 x

46 cm) and housed in a climate-controlled environment at 26-30° C with a 12:12 light:dark cycle. The snakes were provided water *ad libitum* in a small bowl and fed two mice (25 - 40 g total) or, in the case of *N. annulifera*, a small rat every two weeks.

Threat conditions.–Each snake was subjected to three threat conditions in a randomly-assigned but balanced order. The protocols described here were approved by the Institutional Animal Care and Use Committee of Loma Linda University.

For the low threat condition, each snake was placed in an arena (122 x 122 x 76 cm for *C. rhodostoma* and *B. atrox*; 244 x 244 x 122 cm for *N. annulifera*) and allowed 5 min to acclimate. A human-scented, human limb model (500 mL saline-filled glove warmed to 37° C and rubbed against the investigator's bare hands and arms) suspended from a snake stick was lowered into the arena. The model was moved repeatedly toward the snake, with rapid lunges stopping just short of contact. This sequence was repeated until a bite was delivered or until termination of the trial (ca. 15 min). Each trial was videotaped (Sony Hi-8 digital camcorder, 30 fields/sec) from above for subsequent field-by-field playback analysis. For some trials, we failed to capture clear images of the bite. After the snake bit, the model was removed from the arena and gently rocked to mix venom with saline. A sample of the saline was transferred to a 10 mL plastic test tube with a snap-top cap, which was then sealed with parafilm, labeled, and frozen (-20 C) for subsequent analysis.

For the medium threat, conditions were identical to low-threat except that each series of lunges with the human limb model ended in brief contact with the snake, often momentarily pinning the head or other portions of the snake. For the investigator's safety, the cobras were sometimes grasped by the tail during presentation. Samples were treated identically and the same dependent measures were obtained.

For the high threat (venom extraction), each snake was grasped firmly behind the head and mid-body and its mouth placed at the edge of a plastic-covered (Ziploc Sandwhich Bag) beaker until the snake initiated a bite. After the bite, 100 mL of saline was added to the venom and mixed gently. An aliquot was then sealed in a 10 mL test tube that was labeled and frozen for subsequent analysis.

Videotape review.–The variables recorded for the first two conditions (low and medium threat) included time to bite (duration of harassment preceding the bite, in seconds), location of bite on the model (fingers vs. palm), frequency of multiple bites or multiple jaw contractions during the bite, and duration of fang contact with the model (nearest 0.03 sec). Any field in which the fangs appeared to be in contact with the glove was considered to be "contact." For the third condition (high threat), we recorded the time between contact with the parafilm and biting (duration of harassment, in seconds), frequency and duration of independent venom pulses from each fang (nearest 0.03 sec), and number of jaw contractions (associated with fang contractions of viperids) during venom flow. Venom pulses were either bilateral (venom expulsed from both fangs during

a single jaw contraction) or unilateral (venom expulsed from a single fang during a jaw contraction). Pulses were numbered consecutively by their association with consecutive jaw contraction; thus, if the first jaw contraction involved a unilateral pulse from the left fang, we deemed this "left fang pulse 1," and if the second jaw contraction resulted in a bilateral pulse, we deemed this "left fang pulse 2" and "right fang pulse 2." The total duration of venom flow was calculated as the sum of all pulses from each of the two fangs divided by two. Often, one fang expelled venom for a greater duration than the other. We calculated flow differential as the difference in venom flow duration (summed for all pulses) between the two fangs.

Venom measurements.–A total protein assay (Coomassie Protein Assay, 1-25 µg/mL protocol, Pierce Chemical Co.) was performed on all venom samples to determine the dry mass of venom (mg) expended by the snakes. Seven control gloves were filled with 500 mL PBS and each was injected (by tuberculin syringe and needle) with a different amount of venom (0, 10, 20, 30, 40, 50, and 60 mg *C. atrox* venom; Kentucky Reptile Zoo) diluted in 1 mL of phosphate-buffered saline at pH 7.4 (Hayes et al., 1992). Control gloves and their samples were treated in a manner identical to the experimental gloves, including handling with bare hands to transfer human scent to glove exterior. Triplicate samples of the venom standards (from control gloves) and experimental samples (from snake-bitten gloves) were diluted to an appropriate concentration and assayed together in 96-well microtitre plates (Corning, cat. # 430247). Absorbance values (570 nm) from the control gloves were used to generate a standard curve. The standard curve was then used to estimate the mass of venom (mg) injected by

snakes using linear regression. The coefficients of determination for the standard curves indicated the high reliability of the assay (r^2 = 0.920 - 0.996).

For each snake in the high threat condition (venom extractions), we recorded the mass of venom expended (nearest milligram, dry mass), the venom flow rate (venom expended / duration of all pulses summed from both fangs), and venom expended per pulse (venom expended / number of all pulses summed from both fangs). Because flow rates varied within and between individual pulses from the same fang, typically beginning with a steady stream but then tapering off to a trickle, these values were regarded as the average rate of venom flow during the entire duration of venom expulsion.

Data analyses.–All data were analyzed using SPSS 13.0 for Windows. The distribution and variance of data were inspected to determine which statistical tests were appropriate. We relied primarily on general linear models (GLMs), for which the latency to bite, duration of fang contact, mass of venom expended, and venom flow differential had to be rank-transformed to meet parametric assumptions (Mertler and Vannatta, 2004). We also used *t*-tests and Pearson correlations (*r*). Effect sizes–the approximate proportion of variance explained by a dependent variable or interaction–were computed as eta-square (η^2) values for single-factor models, partial η^2 for models having multiple independent variables, and r^2 for bivariate correlations. When the partial η^2 values for main effects and interactions exceeded 1.0, we adjusted these by dividing each partial η^2 by the sum of all partial η^2 values. When deemed appropriate, we also used a number of nonparametric tests (Conover, 1999),

including Cochran's Q, Cramer's V, McNemar test, and Spearman's correlations (r_s). Both V and r_s^2 were interpreted as effect sizes. Alpha levels of 0.05 were used for all tests.

RESULTS

Proportion of stimulus presentations eliciting bite.-The species differed in their biting responses (Table 3-1). Cobras were less likely to bite than the other species in both low-threat (30% of cobras versus 90% for each of the other species; Cramer's $V = 0.62$, $P = 0.003$) and medium-threat (70% of cobras versus 100% for other species; $V = 0.47$, $P = 0.036$) conditions. All snakes bit in the high-threat condition. When bites by the three species were pooled, the difference among the conditions (Cochran's $Q = 14$, asymptotic $P = 0.001$) confirmed that likelihood of biting corresponded to level of threat, though cobras were largely responsible for this relationship. Unfortunately, the fact that only three cobras bit in the low-threat condition resulted in a small sample size for this species in analyses of some of the following bite and venom variables.

Latency to bite.–A 3 x 3 (species x threat condition) mixed analysis of variance (ANOVA), with species treated as a between-subjects factor and threat as a within-subjects factor, revealed that the average time to bite declined significantly with increasing threat ($F_{2,34} = 13.28$, $P < 0.001$, partial $\eta^2 = 0.40$; Table 3-1, Fig. 3-1). The three species also differed in latency to bite, with *C. rhodostoma* requiring the least harassment before biting and *N. annulifera* taking the longest to bite ($F_{2,17} = 6.01$, $P = 0.011$, partial $\eta^2 = 0.38$). The interaction of

species and threat approached significance ($F_{4,34}$ = 2.62, P = 0.052, partial η^2 = 0.20), suggesting that the species differences at lower threat levels did not exist at the highest threat level (venom extraction).

Location of bite.–Bites by the three species were similarly distributed among the two glove locations for low-threat (53% to fingers and 47% to hand; Cramer's V = 0.40, P = 0.26; N = 17) and medium-threat bites (28% to fingers, 72% to hand; V = 0.05, P = 0.97; N = 25; Table 3-1). When bites by the three species were pooled, there was no difference in distribution of bites between the two threat conditions (McNemar test, exact P = 0.29; N = 16).

Jaw contractions.–Multiple jaw contractions were observed in 19 (27.1%) of the 70 bites recorded (Table 3-1). After collapsing number of contractions into two categories (single versus multiple contractions), separate tests of asymmetry (3 species x 2 jaw contraction categories) for each of the three threat conditions indicated that the three species did not differ in proportion of bites involving multiple crunches (low threat: Cramer's V = 0.47, P = 0.16, N = 17; medium threat: V = 0.36, P = 0.20, N = 25; high threat: V = 0.41, P = 0.09, N = 29). When the three species were pooled, there was a significant difference among the three threat conditions in the proportion of bites involving multiple jaw contractions (Cochran's Q = 6.0, P = 0.05). Multiple jaw contractions were more frequent for high-threat than the other conditions

Fang contact and venom flow.–Although duration of fang contact (low- and medium-threat conditions) and venom flow (high-threat conditions) represented different measures (the latter is normally accomplished within time constraints of

the former; Hayes, 2007, Herbert, 1998, Young et al, 2001a), they are closely associated and were considered together here as a single dependent variable: "fang contact." A 3 x 3 (species x threat condition) mixed ANOVA showed that fang contact was similar among the three threat conditions ($F_{2,24}$ = 1.44, P = 0.26, partial η^2 = 0.11; Table 3-1, Figure 3-2). However, the three species differed, with cobras exhibiting bites of much greater duration ($F_{2,12}$ = 8.92, P = 0.004, partial η^2 = 0.60). There was no interaction of species and threat ($F_{4,24}$ = 0.71, P = 0.59, partial η^2 = 0.11), suggesting that the species differences were consistent among the three threat conditions.

The number of jaw contractions was positively correlated with duration of fang contact in the low-threat (Spearman's r_s^2 = 0.30, P = 0.024, N = 17) and medium-threat (r_s^2 = 0.20, P = 0.024, N = 25) conditions, but not in the high-threat condition (r_s^2 = 0.05 , P = 0.24, N = 28).

Venom flow differential between the two fangs could be determined only from the venom extractions. A one-way ANOVA indicated similarity among the three species ($F_{2,25}$ = 2.57, P = 0.097, partial η^2 = 0.17). Venom flow differential in all three species was close to 50% of total duration of venom flow (Table 3-1), indicating substantial variation between the right and left fangs in venom flow during a typical venom extraction bite.

Venom expended.–A 3 x 3 (species x threat condition) mixed ANOVA confirmed that the amount of venom injected increased significantly with higher levels of threat ($F_{2,36}$ = 37.48, P < 0.001, partial η^2 = 0.62; Table 3-1, Fig. 3-3). The three species expended similar quantities of venom ($F_{2,18}$ = 2.71, P = 0.093,

partial $\eta^2 = 0.21$), though the effect size was relatively large. There was no interaction of species and threat ($F_{4,36} = 1.93$, $P = 0.13$, partial $\eta^2 = 0.16$), indicating that the three species showed similar responses at different levels of threat. To make multiple comparisons among the three threat conditions, data were pooled across species (because no species differences existed) and reanalyzed by Bonferroni-adjusted paired *t*-tests. Venom expenditure was significantly greater for the high-threat condition compared to the others ($P < 0.001$), but the low- and medium-threat conditions were similar ($P = 0.55$).

There was no significant correlation between venom flow differential and venom expended (Spearman's $r_s^2 = 0.11$, $P = 0.10$).

Individual venom pulses (high-threat only).–The pattern of venom pulses from the fangs varied considerably in number and synchrony. Of the 28 snakes with adequate video records for quantifying video expulsion, 19 (68%) gave no more than one pulse from one or both fangs, seven (25%) gave two pulses from at least one fang, and two (7%) gave three or more (maximum of five) pulses from at least one fang (Table 3-2). The proportion of individuals giving multiple pulses was statistically similar among the species (*C. rhodostoma*: 56%; *B. atrox*: 30%; *N. annulifera*: 11%; Cramer's $V = 0.38$, $P = 0.13$, N = 28). Although only one *N. annulifera* gave multiple pulses, the five pulses from a single fang exceeded the maximum of three pulses from the other species. Jaw contractions were not always accompanied by venom pulses, as single pulses were delivered in one extraction with two jaw contractions by a *C. rhodostoma* and in another extraction with three jaw contractions by an *N. annulifera*. Twenty-four (61.5%) of

the 39 first, second, and third venom pulses recorded were bilateral, with the remainder being unilateral (Table 3-3). There was no difference in the proportion of bilateral venom pulses between species (Cramer's $V = 0.32$, $P = 0.23$).

Venom pulse characteristics were similar for the right and left fangs, suggesting lack of side dominance in venom expulsion (Table 3-4). When duration of venom flow (rank-transformed) during the first pulse was subjected to a 2 X 3 mixed ANOVA treating fang (right vs. left) as a within-subjects factor and species as a between-subjects factor, there was no difference between left (0.29± 0.05 sec, N = 25; pooled across species) and right (0.24±0.03 sec, N = 23; pooled across species) fangs ($F_{1,17} = 0.04$, $P = 0.84$, partial $\eta^2 = 0.002$). However, there was a significant difference among the three species ($F_{2,17} = 7.25$, $P = 0.005$, partial $\eta^2 = 0.46$), with multiple comparisons showing that mean pulse duration of *C. rhodostoma* (0.23±0.04 sec, N = 15; pooled for both fangs) was similar to the other species, but *N. annulifera* (0.43±0.09 sec, N = 14) was significantly greater than *B. atrox* (0.17±0.01 sec, N = 19; c.f., "fang contact" in high-threat of Fig. 2). The likelihood of multiple pulses was also similar for the two fangs, as five (17.9%) of the 28 snakes gave multiple pulses from the left fang and eight (28.6%) gave multiple pulses from the right fang (pooling independent and related data, Binomial exact $P = 0.58$). When all pulses were assumed to be independent (again pooling independent and related data), the total number of pulses from the left (33) and right (34) fangs was similar (Binomial asymptotic $P = 1.00$).

The duration of venom flow was similar for the first and second pulses. Because only nine snakes delivered two or more pulses, we pooled data across the three species. When pulses were summed for both fangs and divided by two to represent mean duration of venom flow per fang, the durations were similar for the first (0.23±0.04 sec, N = 28, including all snakes) and second (0.32±0.07 sec, N = 9) pulses, though analysis (of rank-transformed data) was limited to the nine matched pairs (paired $t_8 = 1.85$, $P = 0.10$, N = 9). Of these nine snakes, the durations of first and second pulses were not associated (Spearman's $r^2 = 0.03$, $P = 0.65$), demonstrating strong independence between successive pulses. Seven (78%) of the first pulses and four (44%) of the second pulses were bilateral; this difference was not significant (McNemar test, exact $P = 0.25$), although a trend for increased proportion of unilateral bites seemed evident with increasing number of pulses (Table 3-3). The additional pulses delivered by two of the snakes were decidedly brief (third pulses = 0.03 and 0.23 sec; fourth pulses = 0.03 and 0.07 sec; fifth pulse = 0.03 sec), suggesting that pulse duration diminished with increasing number of pulses. The duration of successive pulses from the same fang (N = 13 fangs of nine individuals) was consistent (less than two-fold difference) in eight cases but varied substantially (up to nine-fold) in five cases, again demonstrating strong independence between successive pulses. Among all pulses, pulse duration varied from 0.03-1.00 sec.

Venom flow rates.–A one-way ANOVA comparing venom flow rates (rank-transformed) among the three species found no significant differences (*C. rhodostoma*: 176±52 mg/sec; *B. atrox*: 272±86 mg/sec; *N. annulifera*: 383±98

mg/sec; $F_{2,24}$ = 2.16, P = 0.14, partial η^2 = 0.15; Table 3-1). However, a similar one-way ANOVA comparing venom expended per pulse (rank-transformed) revealed significant differences among the species ($F_{2,24}$ = 3.56, df = 24, P = 0.044, η^2 = 0.23), with Cobras giving the highest venom per pulse (Table 3-1). Tukey's multiple comparisons indicated that *C. rhodostoma* gave significantly less venom per pulse than *N. annulifera*. When all snakes were pooled (N = 27), Pearson correlation analyses showed a strong positive relationship among all three venom variables (venom expended, venom flow rate, and venom expended per pulse, all rank-transformed; r^2 = 0.34-0.68, all Ps ≤ 0.001). Thus, the quantities of venom expended (in total and per pulse) were strongly associated with venom flow rates. Correlations of these variables with total pulse duration (sum of all pulses, rank-transformed) yielded contrasting patterns. First, the mass of venom expended was positively but not significantly associated with total pulse duration (r^2 = 0.05, P = 0.24). After removing three statistical outliers, the positive relationship was significant (r^2 = 0.17, P = 0.048). Second, the venom flow rate was negatively associated with total pulse duration (r^2 = 0.33, P = 0.002). Thus, relatively lengthy pulses had slower flow rates, presumably reflecting the tapering off of venom flow with a protracted pulse. Finally, venom per pulse was independent of total pulse duration, as confirmed by the negative but very weak relationship (r^2 = 0.02; P = 0.49).

DISCUSSION

This study examined the effect of threat intensity on defensive bites by venomous snakes. We found that several behaviors associated with striking, including the quantity of venom expended, differed among the three levels of threat tested. Collectively, the evidence suggests that snakes assess risk and modulate their behaviors, including venom expenditure, accordingly. Moreover, the analyses of venom expulsion suggest that differences in venom expenditure result from variation in number of pulses and/or duration of venom flow, presumably regulated by venom gland contraction and under central nervous system control of the snake.

Risk assessment: striking and biting.–Snakes were more likely to bite and did so more quickly at higher levels of threat, which would be consistent with risk assessment. Although the two viper species generally bit in all of the threat conditions, the cobras were particularly reticent to bite in the low- and medium-threat conditions. The two viper species were also quicker to bite than the cobras. However, regardless of species, the latency to bite decreased significantly with threat level. These findings were consistent with earlier studies suggesting that snakes are reluctant to bite until a threshold level of threat exists. Defensive behaviors such as escape, threat display (elevated head, rattling, body inflation, mouth-gaping), bluff striking, and/or head-hiding often precede biting (Duvall et al., 1985; Goode and Duvall, 1989; Gibbons and Dorcas, 2002; Rowe and Owings, 1990; Shipman, 2002; Hayes, 2007), indicating that venomous snakes generally use their venom only as a last resort. Other aspects of biting

did not vary with threat level, including location of the bite on the model human limb and duration of fang contact or venom flow. Thus, once the bite was elicited, some of the primary kinematics of biting were similar regardless of threat level. We were not able to compare the finer kinematics of biting, such as fang movements and angles of fang penetration (Young et al., 2001a,b, 2003).

Risk assessment: venom expenditure.–In the context of venom metering, the most important finding was that snakes delivered different quantities of venom depending on level of threat. Venom expenditure was statistically similar for bites in the low- and medium-threat conditions, which were elicited from unrestrained snakes by model human limbs (saline-filled gloves). However, the snakes injected substantially more venom in the high-threat condition, when they were physically grasped by the investigator and presented a target (membrane-covered beaker) to bite voluntarily. Like other authors, we considered the latter condition to be one of last resort for the snake, i.e., all defensive tactics up to the point of being grasped had failed to deter or end the confrontation with a "predator" (or antagonist; c.f. Hayes et al., 2002). At this point, any costs associated with use and replenishment of venom (McCue, 2006) might be outweighed by the benefit of inducing a painful, debilitating bite with maximum venom injection.

Several questions need to be addressed regarding the differences in venom expenditure. First, how did the snakes deliver more venom during bites in the high-level threat condition? Two possibilities exist. Multiple jaw contractions were observed during bites in all three conditions; however, the greater number

observed for the high-threat condition likely increased the amount of venom injected. Fang contact duration during the low- and medium-threat conditions was equivalent to the duration of venom flow in the high-threat condition; however, because fang contact included both engagement and disengagement of fangs, with venom flow likely occurring during only a small portion of fang contact time (Young et al., 2001a), we believe that venom flow duration was actually longer during the high-threat bites. Venom flow during high-threat bites may have involved greater force and/or multiple pulses (see below). Second, do the differences in venom expenditure constitute venom-metering–a decision made by the snake as to how much venom to inject? We believe the answer to this is "yes." Although kinematic differences could account for venom differences, there is growing evidence that snakes can control duration of both fang engagement and venom flow, as demonstrated in this study.

Venom expulsion.—The emerging picture from studies of venom expulsion from the fangs suggests both remarkable control and functional independence of each of the two separate venom delivery systems—the right and left systems. Fang movements (in viperids) and venom expulsion by the two systems, although coordinated during a bite, often differed substantially in initiation and duration. One or both systems were capable of delivering multiple pulses, some synchronous and others asynchronous. Each of the consecutive pulses was always associated with (sometimes subtle) separate jaw contractions, suggesting that venom expulsion is coupled to coordinated jaw movements that drive the fangs into the target. Within a single jaw contraction,

we found no correlation between the right and left systems in duration of venom flow and between consecutive pulses from the same fang, reinforcing our view of remarkable independence between the right and left venom delivery systems.

Our analyses of venom expulsion here and elsewhere support our view that snakes can control venom expenditure by means of differential venom gland contraction (Hayes, 2007; Hayes et al., in press). Within the envenomation systems, two key components that regulate venom flow include venom gland contraction, which provides the only motive force for venom flow, and fang sheath compression, which exposes the fangs and displaces an internal membrane that removes the internal block to venom flow. Recent papers have questioned the importance of gland contraction, emphasizing instead priority of fang sheath displacement, influenced largely by target features and largely beyond the snake's control (e.g., Young et al., 2003; Young, 2007; Young and Kardong, 2007). Unfortunately, the empirical data supporting this view were derived from flawed experimental designs that led to invalid comparisons and conclusions (see Hayes, 2007; Hayes et al., in press). Although the fang sheath clearly serves as a gate for permitting venom flow, no evidence exists that minute differences in fang sheath compression during biting can significantly alter venom flow. The quantity of venom ejected from the fangs will be a product of both force and duration of venom flow, properties more effectively regulated by venom gland contraction. Despite injection through the same target (the membrane covering the beaker), the venom pulses varied substantially in duration between the right and left sides, and between successive pulses. Differences in fang

sheath compression, which we did not measure, seem highly unlikely to account for such variation in pulse duration. Our impression was that fang sheath compression was at full extent during most pulses examined. Because the snakes were grasped by the investigator's right hand, bias could be expected from greater pressure exerted on one fang sheath compared to the other, yet no differences were seen in venom flow between the two sides.

The negative relationship between venom flow rate and total pulse duration confirmed our visual impression (Herbert, 1998) that force of venom expulsion can vary within a single pulse, particularly as it diminishes toward the end of a pulse. We frequently observe this tailing off of venom flow without a change in fang sheath displacement, reinforcing our view that fang sheath displacement is not a primary determinant of pulse duration. The significant positive relationship between venom expended and total pulse duration reinforces our view that flow duration is a critical determinant of total venom expended.

Results of the present study complement those of Herbert (1998), Hayes (2007), and Hayes et al. (in press) on other snake species, underscoring the functional convergence between the venom delivery systems of viperid and elapid snakes (Young and Kardong, 2007).

Both elapids (cobras in this study) and viperids (rattlesnakes and cottonmouths; Herbert, 1998; Hayes, in press) exhibit independent control of each fang. Representatives of both families are capable of delivering bilateral (from both fangs) or unilateral (from one fang) venom pulses during a bite. Both

can deliver multiple venom pulses of independently-varying duration with a brief interval between successive pulses. In both groups, individual pulses are associated with jaw contractions that, in viperids, also involve fang retraction. In the present study, the elapid (*Naja annulifera*) exhibited venom pulses of longer duration than the two viperid species. Consequently, the venom expended per pulse was also greatest for the elapid, but the rate of venom flow was similar. Venom flow rates correspond to body size, particularly the diameter of venom ducts and fangs (Herbert, 1998; Hayes et al., in press). In the present study, size differences between the three species were apparently insufficient to result in different venom flow rates. Despite well-documented differences in their venom delivery systems (Jackson, 2003; Young and Kardong, 2007), there are considerable similarities between venom delivery characteristics for the two snake families.

LITERATURE CITED

Caro, T. 2005. Chapter 10. Antipredator Defenses in Birds and Mammals. University of Chicago Press, Chicago, Illinois.

Conover, W. J. 1999. Practical Nonparametric Statistics (3rd ed). John Wiley & Sons, Inc., New York.

Duvall, D., M. B. King, and K. J. Gutzwiller. 1985. Behavioral ecology and ethology of the prairie rattlesnake. Nat. Geo. Res. 6:457-469.

Gibbons, J. W., and M. E. Dorcas. 2002. Defensive behavior of cottonmouths (*Agkistrodon piscivorus*) toward humans. Copeia 2002:195-198.

Goode, M. J., and D. Duvall. 1989. Body temperature and defensive behavior of free-ranging prairie rattlesnakes, *Crotalus viridis viridis*. Anim. Behav. 38:360-362.

Graves, B. M. 1989. Defensive behavior of female prairie rattlesnakes, *Crotalus viridis*, changes after parturition. Copeia 1989:791-794.

Hayes, W. K. 2007. The snake venom-metering controversy: levels of analysis, assumptions, and evidence. In press *in* W. K. Hayes, K. R. Beaman, M. D. Cardwell, and S. P. Bush (eds.), The Biology of Rattlesnakes. Loma Linda University Press, Loma Linda, California.

Hayes, W. K., I. I. Kaiser, and D. Duvall. 1992. The mass of venom expended by prairie rattlesnakes when feeding on rodent prey. Pp. 383-388 *in* J. A. Campbell and E. D. Brodie, Jr., eds. Biology of the Pitvipers. Selva, Tyler, Texas.

Hayes, W. K., S. S. Herbert, G. C. Rehling, and J. Gennaro. 2002. Factors that influence venom expenditure in viperids and other snake species during predatory and defensive contexts. Pp. 207-233 *in* G.W. Schuett, M. Höggren, M. E. Douglas, and H. W. Greene (Eds.), Biology of the Vipers. Eagle Mountain Publ., Eagle Mountain, Utah.

Herbert, S. S. 1998. Factors influencing venom expenditure during defensive bites by cottonmouths (*Agkistrodon piscivorus*) and rattlesnakes (*Crotalus viridis*, *Crotalus atrox*). M.S. Thesis, Loma Linda University, Loma Linda, California.

Jackson, K. 2003. The evolution of venom-delivery systems in snakes. Zool. J. Linn. Soc. 137:337–354.

Lima, S. L. and Dill, L. M. 1990. Behavioral decisions made under the risk of predation: a review and prospectus. Can. J. Zool. 68: 619–640.

Lima, S. L., N. C. Rattenborg, J. A. Lesku, C. J. Amlaner. 2005. Sleeping under the risk of predation. Anim. Behav. 70:723-736.

McCue, M. D. 2006. Cost of producing venom in three North American pitviper species. Copeia 2006:818-825.

Mertler, C. A., and R. A. Vannatta. 2004. Advanced and Multivariate Statistical Methods: Practical Application and Interpretation. 3rd ed. Pyrczak Publishing, Los Angeles, California.

Rehling, G. C. 2002. Venom expenditure in multiple bites by rattlesnakes and cottonmouths. M.S. Thesis, Loma Linda University, Loma Linda, California.

Rowe, M. P., and D. H. Owings. 1990. Probing, assessment, and management during interactions between ground squirrels and rattlesnakes. Part 1: risks related to rattlesnake size and body temperature. Ethology 86:237-249.

Shine, R., L. Sun, M. Fitzgerald, and M. Kearney. 2002. Antipredator responses of free-ranging pit vipers (*Gloydius shedaoensis*, Viperidae). Copeia 2002:843-850.

Shipman, P. A. 2002. The evolution of antipredator behavior in snakes: ultimate and proximate determinants. Ph.D. Dissertation, Oklahoma State University, Stillwater, Oklahoma.

Taylor, R. A., M. H. Persons, and A. L. Rypstra. 2005. The effect of perceived predation risk on male courtship and copulatory behavior in the Wolf Spider *Pardosa milvina* (ARANEAE, LYCOSIDAE). J. Arachnol. 33:76-81.

Whitaker, P. B., K. Ellis, and R. Shine. 2000. The defensive strike of the eastern brownsnake, *Pseudonaja textilis* (Elapidae). Funct. Ecol. 14:25-31.

Whitaker, P. B., and R. Shine. 1999. Responses of free-ranging brownsnakes (*Pseudonaja textilis*, Elapidae) to encounters with humans. Wildlife Res. 26:689-704.

Young, B. A. 2007. Perspectives on the regulation of venom expulsion in snakes. In press *in* W. K. Hayes, K. R. Beaman, M. D. Cardwell, and S. P. Bush (eds), The Biology of Rattlesnakes. Loma Linda University Press, Loma Linda, California.

Young, B. A., M. Blair, K. Zahn, and J. Marvin. 2001a. Mechanics of venom expulsion in *Crotalus*, with special reference to the role of the fang sheath. Anat. Rec. 264:415-426.

Young, B. A., M. Phelan, J. Jaggers, and N. Nejman. 2001b. Kinematic modulation of the strike of the western diamondback rattlesnakes (*Crotalus atrox*). Hamadryad 26:316-349.

Young, B. A., M. Phelan, M. Morain, M. Ommundsen, and R. Kurt. 2003. Venom injection in rattlesnakes (*Crotalus*): peripheral resistance and the pressure-balance hypothesis. Can. J. Zool. 81:313-320.

Young, B. A., and K. V. Kardong. 2007. Mechanical and physiological influences on venom expulsion in the western diamondback rattlesnake, *Crotalus atrox*. J. Exp. Zool. 307A:18-27.

Table 3-1. Dependent measures associated with defensive bites by three snake species (*Calloselasma rhodostoma*, CR; *Bothrops atrox*, BA; *Naja annulifera*, NA) during three levels of threat.

Independent Variable	Low Threat			Moderate Threat			Venom Extraction		
	CR	BA	NA	CR	BA	NA	CR	BA	NA
Snakes that bit: % (N)	90 (10)	90 (10)	30 (10)	100 (10)	100 (10)	70 (10)	100 (10)	100 (10)	100 (10)
Latency to bite (min): $\bar{x}$ ± 1 SE (N)	0.9 ± 0.6 (9)	5.4 ± 2.1 (8)	11.7 ± 3.2 (3)	0.6 ± 0.2 (9)	3.4 ± 1.0 (8)	10.6 ± 0.7 (3)	0.2 ± 0.1 (9)	0.3 ± 0.1 (8)	0.4 ± 0.2 (3)
Bite location: % to hand (N)	44 (9)	67 (6)	0 (2)	70 (10)	75 (8)	71 (7)	--	--	--
Jaw contractions: range, % multiple (N)	1-2 11 (9)	1-6 50 (6)	1 0 (2)	1 0 (9)	1-6 25 (8)	1-3 29 (7)	1-3 66 (9)	1-2 30 (10)	1-5 20 (10)
Fang contact/venom flow (sec): $\bar{x}$ ± 1 SE (N)	0.34 ± 0.11 (8)	0.99 ± 0.61 (5)	1.20 ± 0.03 (2)	0.24 ± 0.09 (8)	0.25 ± 0.05 (5)	0.90 ± 0.13 (2)	0.27 ± 0.05 (8)	0.16 ± 0.13 (5)	0.57 ± 0.18 (2)
Venom injected (mg): $\bar{x}$ ± 1 SE (N)	13.0 ± 4.0 (9)	25.2 ± 13.1 (8)	8.3 ± 3.0 (3)	28.2 ± 22.5 (9)	21.8 ± 3.7 (8)	34.4 ± 3.0 (4)	85.8 ± 30.8 (9)	90.1 ± 17.6 (8)	127.7 ± 24.5 (4)
Venom flow differential (sec): $\bar{x}$ ± 1 SE (N)	--	--	--	--	--	--	0.14 ± 0.04 (9)	0.07 ± 0.03 (10)	0.23 ± 0.08 (9)
Venom flow rate (mg/sec): $\bar{x}$ ± 1 SE (N)	--	--	--	--	--	--	176 ± 52 (9)	272 ± 86 (10)	383 ± 98 (8)

Table 3-2. Proportion of snakes in high-threat condition giving one, two, or three or more venom pulses during a single defensive bite. A pulse is defined as an expulsion of venom through the left, right, or both fangs during a jaw contraction. Sample size (n) is in parentheses.

Species	1 Pulse Only	2 Pulses	3+ Pulses
Calloselasma rhodostoma	44% (4)	44% (4)	10% (1)
Bothrops atrox	70% (7)	30% (3)	0% (0)
Naja annulifera	90% (8)	0% (0)	10% (1)
All snakes	68% (N = 19)	25% (N = 7)	7% (N = 2)

Table 3-3. Proportion of snakes in high-threat condition giving bilateral (from both left and right fangs) versus unilateral (from just one fang) venom pulses for up to three consecutive pulses during a single defensive bite. Sample size (N) is in parentheses.

	Pulse 1		Pulse 2		Pulse 3	
Species	**Bilateral**	**Unilateral**	**Bilateral**	**Unilateral**	**Bilateral**	**Unilateral**
Calloselasma rhodostoma	67 (6)	33 (3)	20 (1)	80 (4)	0 (0)	100 (1)
Bothrops atrox	90 (9)	10 (1)	67 (2)	33 (1)	0 (0)	0 (0)
Naja annulifera	56 (5)	44 (4)	100 (1)	0 (0)	0 (0)	100 (1)
All snakes	71 (20)	29 (8)	44 (4)	56 (5)	0 (0)	100 (2)

Table 3-4. Pulse duration (mean seconds ± 1 SE) for venom pulses during the high threat bites by *Calloselasma rhodostoma*, *Bothrops atrox*, and *Naja annulifera*. L = left fang; R = right fang; AVG = average for all L and R pulses. Sample size (N) in parentheses.

Snake-Extraction	Pulse 1			Pulse 2			Pulse 3		
	L	R	AVG	L	R	AVG	L	R	AVG
Calloselasma rhodostoma	0.31 ± 0.07 (6)	0.18 ± 0.03 (9)	0.23 ± 0.04 (15)	0.17 -- (1)	0.29 ± 0.12 (5)	0.27 ± 0.12 (6)	0.23 -- (1)	--	0.23 -- (1)
Bothrops Atrox	0.14 ± 0.1 (10)	0.20 ± 0.03 (9)	0.17 ± 0.01 (19)	0.19 ± 0.03 (3)	0.18 ± 0.05 (2)	0.19 ± 0.05 (5)	--	--	--
Naja Annulifera	0.44 ± 0.12 (9)	0.41 ± 0.12 (5)	0.43 ± 0.09 (14)	0.03 -- (1)	0.27 -- (1)	0.15 -- (2)	--	0.03 -- (1)	0.03 -- (1)
Overall Mean	0.29 ± 0.05 (25)	0.24 ± 0.03 (23)	0.27 ± 0.08 (48)	0.15 ± 0.03 (5)	0.26 ± 0.10 (8)	0.22 ± 0.09 (13)	0.23 -- (1)	0.03 -- (1)	0.13 -- (2)

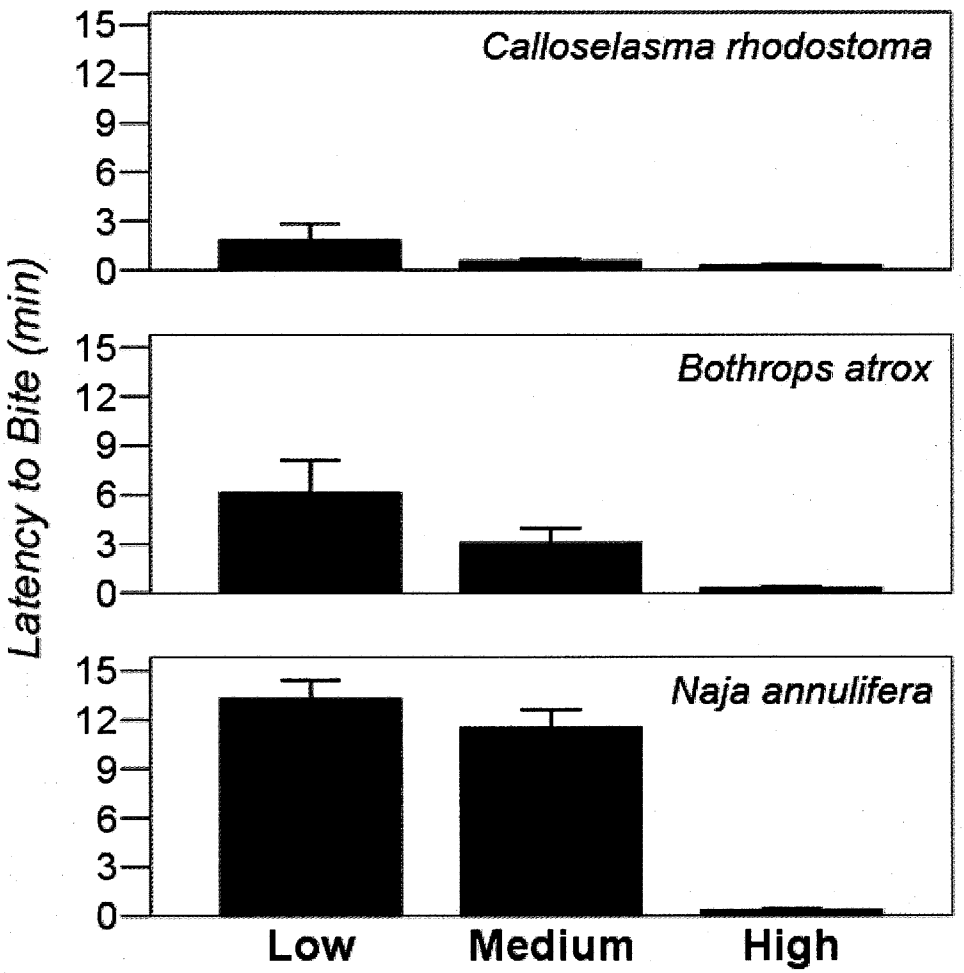


Figure 3-1. Mean (+ 1 S.E.) latency to defensive bites by three snake species during low, medium, and high threat conditions.

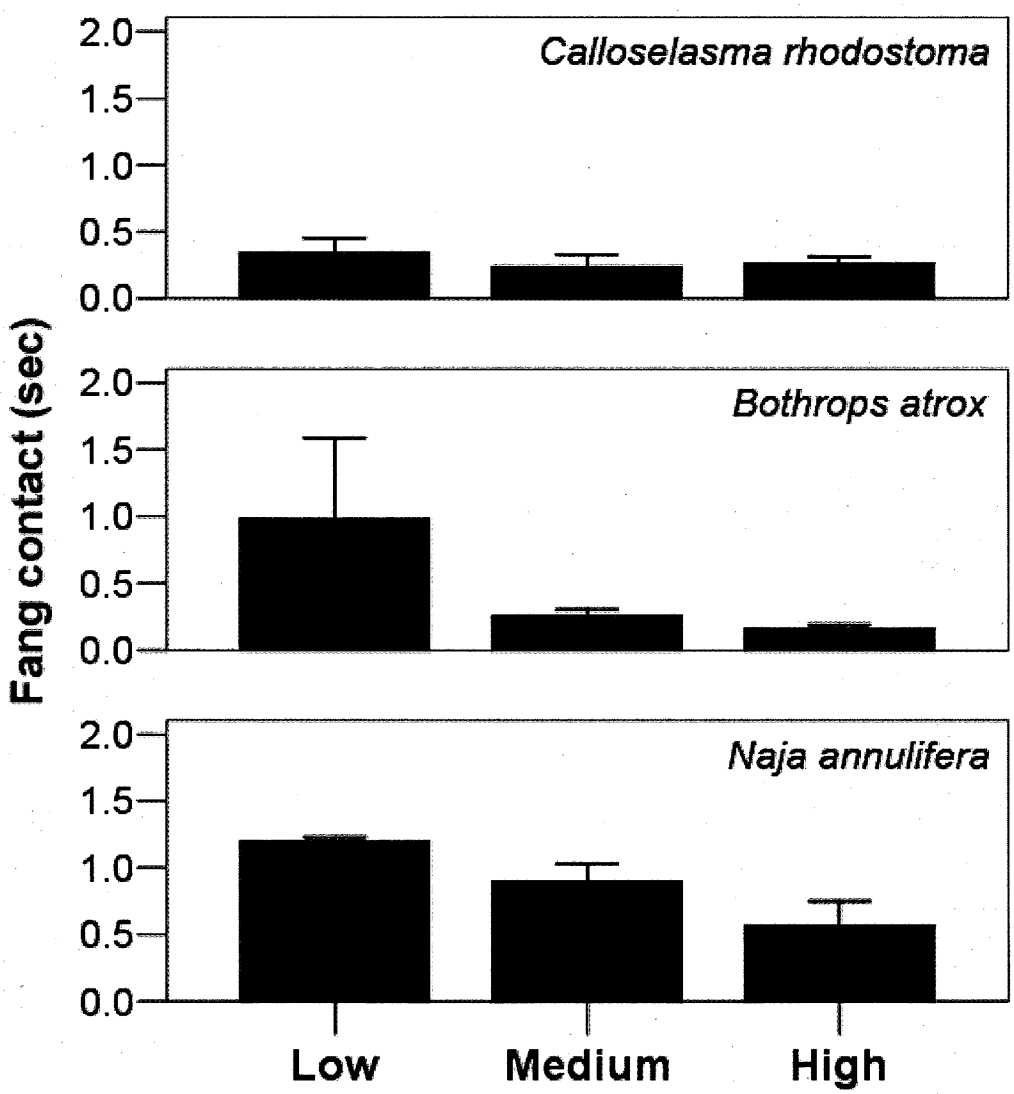


Figure 3-2. Mean (+ 1 S.E.) duration of fang contact by three snake species during low, medium, and high threat conditions

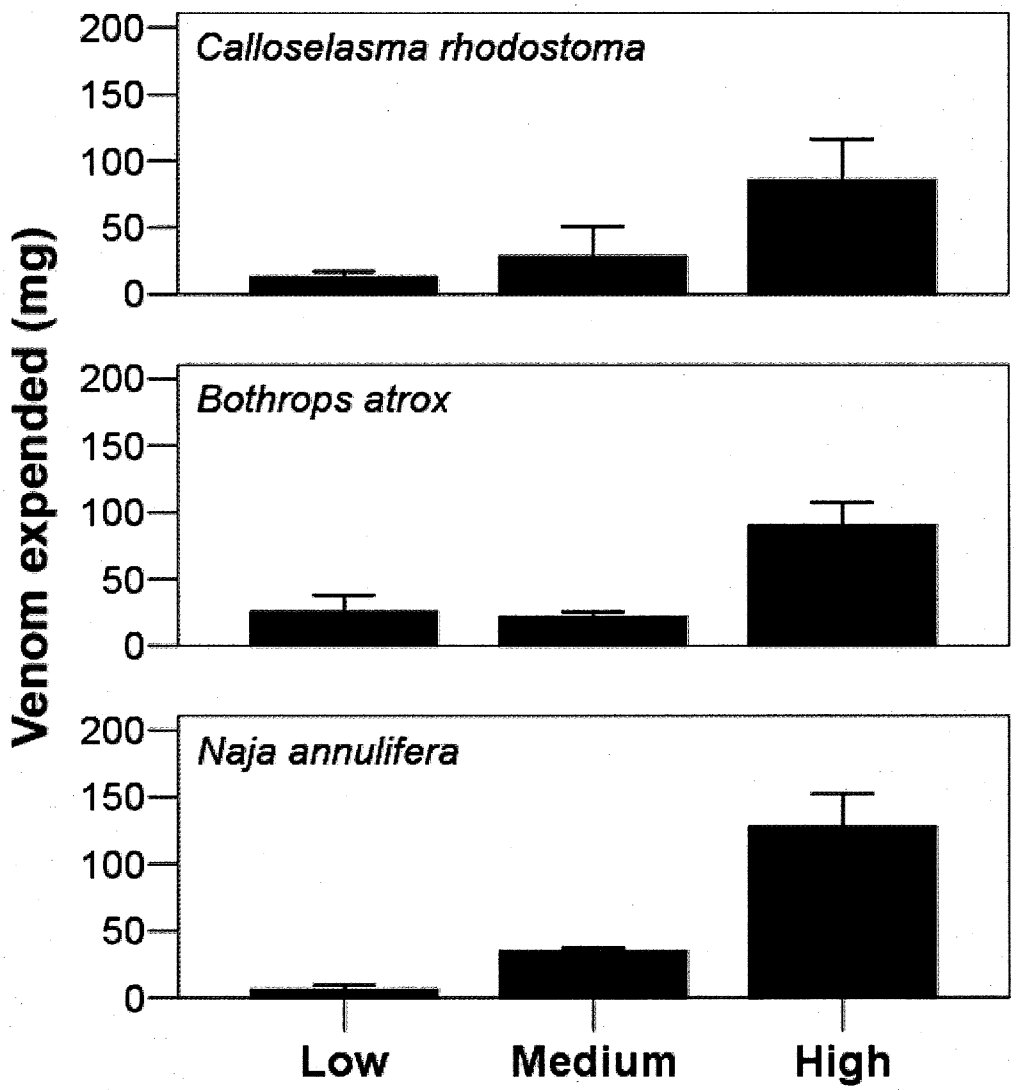


Figure 3-3. Mean (+ 1 S.E.) mass of venom expended by three snake species during low, medium, and high threat conditions.

Chapter IV

Venom Expenditure by Rattlesnakes and Killing Effectiveness in Rodent Prey: Do Rattlesnakes Expend Optimal Amounts of Venom?

Shelton S. Herbert and William K. Hayes

Department of Earth and Biological Sciences, Loma Linda University, Loma Linda, CA 92350 USA

This chapter has been accepted for publication with the following citation:

Herbert, S. S., and W. K. Hayes. 2007. Venom expenditure by rattlesnakes and killing effectiveness in rodent prey: do rattlesnakes expend optimal amounts of venom? In press *in* W. K. Hayes, K. R. Beaman, M. D. Cardwell, and S. P. Bush (eds.), The Biology of Rattlesnakes. Loma Linda University Press, Loma Linda, California.

ABSTRACT

Optimal foraging theory proposes that animals are designed to maximize energy intake while minimizing costs of procurement. Because venom is a limited commodity due to storage constraints and costs of production (metabolic and ecological), venomous animals should be judicious in the amounts they deploy when acquiring food. Here, we considered whether the amount of venom injected by adult Prairie Rattlesnakes *(Crotalus viridis)* into rodents might be optimized in terms of killing effectiveness. The results of experiment 1 supported our prediction that the quantity of venom rattlesnakes typically inject into mice (16 mg) would produce the most rapid incapacitation and death for the least amount of venom. Mice injected by syringe died more quickly with increasing doses of venom up to 15 mg, but those injected with greater quantities did not succumb

more rapidly. The results of experimental 2 supported our prediction that the optimum dose for securing larger rodent prey (rats and hamsters) should be greater than that for smaller prey (mice), matching the pattern of venom expenditure documented in behavioral studies of snakes. Indeed, the larger prey survived longer and increasing doses of venom caused more rapid prey death regardless of prey type; however, variation in time to death was too great to determine optimas for either rats or hamsters. The results add to a growing body of evidence supporting adaptive use of venom by snakes. However, optimality may constrained by numerous factors, including phylogenetic inertia, different optimas for other functions of venom (e.g., prey-marking to relocate prey released after envenomation, enhanced digestion following consumption), competing selection for other traits (e.g., physiology, venom toxicity) and contexts (defensive), and environmental changes that affect both predator and prey populations. We propose that selection can act on cognition (decision-making), favoring different behavioral strategies for deploying varying quantities of venom depending on the target and the context.

INTRODUCTION

Optimality theory has been applied successfully to a wide range of biological problems, including those associated with foraging, reproduction, social behavior, communication, and even molecular and physiological function (e.g., Stephens and Krebs, 1986; Orzack and Sober, 2001; Todorov, 2004; Goodarzi et al., 2005). These studies assume and often demonstrate that

animals, or specific properties thereof, evolve via natural selection to become more efficient. As an effective, though sometimes controversial approach for demonstrating adaptation (Orzack and Sober, 2001), optimality studies frequently help us better understand the ultimate cause(s) and function(s) of a trait in question. Most animals must make decisions about foraging. Because procuring energy is essential for survival and reproduction, natural selection ensures that animals become adept at acquiring resources. In essence, animals seek to maximize energy intake while minimizing costs of procurement, ultimately increasing their lifetime reproductive success (fitness). Examples of decisions to be made include how and where to search for food and what food items to ignore or consume (e.g., Stephens and Krebs, 1986; Perry and Pianka, 1997). Decisions can be influenced by both external (e.g., prey availability, predation risk, habitat structure, social interactions, toxins, distasteful compounds) and internal factors (e.g., age, hunger, sex and reproductive state, learned experiences, dietary preferences, nutritional requirements; Perry and Pianka, 1997).

Many animals rely on venoms to procure food and/or defend themselves. Because venom can be viewed as a limited commodity due to costs of production (metabolic and ecological) as well as storage constraints (Hayes et al., 2002; Hayes, this volume; McCue, in press), venomous animals may be designed to optimize the amounts they deploy when acquiring food. Organisms as simple as anemones and jellyfish appear to regulate their venom via cellular mechanisms that inhibit excess venom expenditure (Thorington and Hessinger,

1998). Spiders, tarantulas, and cone snails are similarly judicious in their use of venom, expending quantities that often correspond to the size and/or escape ability of their prey and sometimes withholding venom when subduing small prey (reviewed by Hayes et al., 2002; Stewart and Gilly, 2005; Hostettler and Nentwig, 2006).

Rattlesnakes serve as an excellent model for studying optimal venom deployment. Numerous studies suggest that they allocate, or meter, different quantities of venom when striking in different contexts (e.g., predatory vs. defensive, or hungry vs. well-fed) or when biting different targets (e.g., different species or sizes of prey; Hayes et al., 2002; Hayes, 2007). Because many snakes, including rattlesnakes, often strike, envenomate, release, and subsequently relocate prey that travel some distance before dying (e.g., Kardong and Smith, 2002), snakes may be unique among venomous animals in making decisions on how much venom to inject before launching a predatory attack (Hayes et al., 2002). Allocation decisions made by other venomous predators studied to date rely on feedback from a struggling prey item. Although the relative quantities of venom injected by rattlesnakes into different prey types are consistent with expectations of adaptive function (Hayes, 2007), we have not considered whether the exact quantities injected are to any extent optimized.

Within the context of feeding, the optimal amount of venom to inject could be influenced by a number of functions that venom serves (reviewed by Hayes et al., 2002; Kardong, 2002). Snake venoms not only immobilize and kill their prey, but also help to relocate prey released immediately after striking (by altering the

scent trail deposited by the fleeing prey) and to accelerate digestion (preventing putrefaction and regurgitation of larger, bulkier prey). By injecting insufficient venom, the snake may fail to secure its meal. The envenomated prey might travel beyond recovery range, deposit an inadequate odor trail for efficient recovery, or take too long to digest. Injecting too much venom would be metabolically wasteful and could leave the snake with inadequate venom to procure additional prey or defend itself. Thus, we expect that selection would favor snakes that dispense optimal amounts of venom when feeding.

The purpose of this study was to explore the possibility that venom expenditure by one well-studied taxon, the Prairie Rattlesnake (*Crotalus viridis*), is at or near optimal performance. Here, we simply considered whether the amount of venom injected by adult rattlesnakes into rodents would correspond to that which would immobilize and kill within an optimal amount of time. We made two predictions regarding the effects of venom on prey. First, because adult Prairie Rattlesnakes typically inject 16 mg of venom in a single bite of an adult mouse (Hayes, 1992a), we predicted that this quantity would provide the most rapid immobilization and death for the least amount of venom. Second, because these snakes inject more venom into larger prey (Hayes, 1995; Hayes et al., 1995, 2002), we predicted that the optimal quantity of venom to inject in larger and/or venom-resistant prey would be greater than that for smaller and/or less-resistant prey.

METHODS

The basic experimental design was to inject varying doses of venom into prey animals and quantify time to immobilization and death. Because rapid immobilization and death of prey are among the primary functions of venom injection by snakes, the use of live rodents was essential for testing hypotheses of venom function. To experimentally control the dose of venom injected, we circumvented natural envenomation of mice intended to be fed to the snakes by artificially injecting the mice with measured quantities of venom. Thus, our methods essentially duplicated what the rodents would have experienced during natural snakebite. All rodents killed by envenomation were subsequently fed to the snakes housed in our research collection. Death by envenomation at natural doses of venom (5-25 mg in mice; Hayes, 1992a) is more rapid and humane than the widely-employed conventional assays of venom toxicity in rodents, which are conducted with minute quantities of venom (generally much less than 1 mg) and measured over a 24 hr period (Sells, 2003). The protocols described here were approved by the Institutional Animal Care and Use Committees of Southern College, Tennessee (for hamsters and mice), and Loma Linda University (for rats and additional mice).

Venom.–Lyophilized venom from South Dakota populations of adult *C. v. viridis* were purchased from the Miami Serpentarium and from the Kentucky Reptile Zoo. The venom was reconstituted in phosphate-buffered saline (PBS, pH = 7.4; see Hayes et al., 1992a).

Prey animals.–We used three different prey animals. Laboratory mice (*Mus musculus*) were raised in our laboratory or purchased from a local supplier. These mice were generic rather than of a particular strain. Generic laboratory rats (*Rattus norvegicus*) and Golden Hamsters (*Mesocricetus auratus*) were also purchased from local suppliers and raised in our laboratory. We used only adults from each group.

Experiment 1: Effects of venom dose on mice.–We evaluated the effects of five venom doses on a single prey species. To assess optimality, we required natural prey that rattlesnakes regularly consume. However, because Deer Mice (*Peromyscus maniculatus*), a major dietary item of *C. v. viridis* (Duvall et al., 1990), and laboratory mice (*Mus musculus*) are similarly affected by *C. v. viridis* venom in terms of time to immobilization and death (Hayes, 1991), we chose to use laboratory mice, which are much easier to acquire and maintain. Mice of both sexes (15-40 g; n = 107) were randomly assigned a single injection of one of five different doses of venom (5, 10, 15, 20, or 25 mg dissolved in 0.5 ml phosphate-buffered saline) administered to the right-lateral, middorsal region. Injections were made by 1 cc tuberculin syringe and 24 gauge needle at a depth of 6-9 mm, which is comparable to the fang lengths of adult *C. v. viridis* (Klauber, 1936). We assumed that with a large sample size the effects of venom injection by a single needle were comparable to the usual delivery via two snake fangs. There was no difference in the mean mass of mice assigned to each of the five doses. Additional control mice injected with only saline did not die or show adverse effects; hence, venom was clearly the cause of effects observed following

injection. Because rattlesnakes ordinarily do not deliver dry bites to mice (Kardong, 1986; Hayes, 1992a) and the need for such controls was irrelevant to the purpose of the study, the 0 mg controls were excluded from all analyses.

Experiment 2: Effects of venom dose on three prey types.–We compared the effects of three venom doses on prey of three classes. For small prey, we used the data acquired from Experiment 1. For larger prey, we used both sexes of lab rats (71-124 g, n = 36) and hamsters (67-140 g, n = 21), respectively. Feeding observations suggested that hamsters live much longer than rats following envenomation; therefore, the hamsters served as a model for venom-resistant prey. Each animal was randomly assigned one of three different venom concentrations (5, 15, and 25 mg in 0.5 ml PBS total volume). There was no difference in the mean mass of rats and hamsters in each group. Injections were performed in a manner identical to those for mice. Again, 0 mg controls in rats and hamsters produced no adverse effects, and these were excluded from analyses.

Effects of envenomation.–Immediately after venom injection, we placed each rodent in its own plastic observation chamber (28 cm x 23 cm). Using a handheld stopwatch, we then recorded time to immobilization (seconds until cessation of locomotion) and time to death (seconds until last visible movement) for each animal. For mice, there is a strong correlation between time to immobilization, time to death, and distance traveled after envenomation (Hayes, 1992a). Any prey item surviving past the pre-determined cutoff (15, 60, 180 min for mice, rats, and hamsters, respectively) was humanely euthanized (by cervical

dislocation). All rodent carcasses were either fed immediately to snakes or were stored frozen, to be fed to snakes at a later time.

Analyses.–For experiment 1 (mice), we used one-way ANOVAs to analyze both dependent measures (time to immobilization, time to death), treating the independent variable (dose, with five levels) as a between-subjects factor. Both dependent measures were rank-transformed to meet assumptions of normality and homoscedasticity. Analyses of both data and ranks gave identical results except for multiple comparisons (Tukey's tests), with a slight difference in conclusions; hence, both results are presented. A similar ANOVA indicated that mice in each group had equivalent mass.

For experiment 2 (mice, rats, hamsters), the dependent measures (time to immobilization, time to death) were subjected to 3 x 3 ANOVAs treating both independent variables (prey type, 3 levels; venom dose, 3 levels) as between-subjects factors. The dependent measures were also rank-transformed, though parametric assumptions still were not strictly met. Multiple comparisons were conducted using Tukey's tests. An additional ANOVA showed that rats and hamsters were equal in mass, and that groups assigned to different doses were also similar in mass.

Tests were conducted using SPSS 12.0 software (Statistical Package for the Social Sciences, Inc., Chicago, Illinois, 2003), with alpha = 0.05. Apart from the 0 mg controls, no data were discarded as outliers. Effect sizes for each test

were obtained as η^2 values, indicating the approximate proportion of variance in the dependent variable explained by an independent variable or interaction.

RESULTS

Experiment 1: Venom effectiveness in mice.–For both time to immobilization ($F_{4,102} = 8.31$, $P < 0.001$, $\eta^2 = 0.25$) and time to death ($F_{4, 102} = 8.47$, $P < 0.001$, $\eta^2 = 0.25$), there was a significant effect of venom dose (statistics are for rank-transformed data). As expected, mice were immobilized and died more quickly with increasing dose of venom injected (Fig. 1). However, Tukey's multiple comparisons of both data and ranks indicated that doses greater than 10-15 mg yielded diminishing returns for hastening immobilization and death (see Fig. 1). Immobilization occurred significantly faster at 10 mg than 5 mg, but doses $\geq$10 mg yielded similar results. Death resulted more quickly at 15 mg than 10 mg, but doses $\geq$15 mg were equivalent. Thus, the optimal dose of venom to inject, producing the most rapid effects with least expenditure of venom, was 10-15 mg. At any given dose, the time to death was at least three-fold greater (range: 3.0-4.7) than the time required for immobilization.

Experiment 2: Venom effectiveness in mice, rats, and hamsters.–The main effect of prey type was significant for both time to immobilization ($F_{2,112} = 21.88$, $P < 0.001$, partial $\eta^2 = 0.28$) and time to death ($F_{2, 112} = 52.13$, $P < 0.001$, partial $\eta^2 = 0.25$). Venom effects corresponded loosely to prey size, with mice succumbing more quickly than the larger prey (Fig. 2). Pairwise comparisons of rank-transformed data indicated that mice < rats = hamsters for immobilization

and mice < rats < hamsters for death. Thus, in spite of their equivalent mass, hamsters were more resistant to the venom than rats.

The main effect of venom dose was also significant for both time to immobilization ($F_{2,112} = 4.98$, $P = 0.008$, partial $\eta^2 = 0.08$) and time to death ($F_{2,112} = 3.97$, $P = 0.022$, partial $\eta^2 = 0.07$), though effect sizes were considerably smaller than those for the main effect of prey type. Animals injected with more venom died more quickly (Fig. 2). However, with the smaller sample sizes for larger prey (rats: N = 12 for each mean; hamsters: N = 6-9 for each mean), multiple comparisons were less informative, with the only pairwise difference being between 5 and 25 mg doses for hamsters. Comparing time to death between the 15 and 25 mg injections, mean latency to death was unexpectedly similar for rats (9.6 and 9.8 min, respectively), but hamsters died in less than half the time at 25 mg (30.1 min) compared to 15 mg (79.1 min). In the rats and hamsters, time to death was roughly 1.3-2.3-fold longer than time to immobilization.

There was no interaction between prey type and venom dose for either time to immobilization ($F_{4,112} = 1.76$, $P = 0.141$, partial $\eta^2 = 0.06$) or time to death ($F_{4,112} = 2.33$, $P = 0.06$, partial $\eta^2 = 0.08$). Thus, the dose-dependent effects of venom on time to immobilization and death were similar, regardless of prey type.

DISCUSSION

The results of this study, particularly those of experiment 1 involving mice, support the view that adult Prairie Rattlesnakes expend a near-optimal quantity

of venom when procuring adult rodent prey. Such an optimum represents a balance between energy expended (including venom synthesis and storage and relocation of dispatched prey) and energy procured from the prey. Whereas increasing venom doses caused increasingly rapid immobilization and death of adult mice, quantities greater than 10-15 mg did not significantly hasten immobilization and death. Delivery of more than 15 mg provided diminishing returns as the dependent measures (immobilization and death) presumably approached asymptotes. Although adult Prairie Rattlesnakes are capable of expending much more or much less venom, they inject an average of 16 mg venom into adult mice (Hayes, 1992a), which appears to be close to an optimal quantity.

The results of experiment 2 are more difficult to interpret because of the smaller sample sizes and correspondingly reduced statistical power. However, several conclusions can be drawn. First, the significant effects of prey type (for both immobilization and death) confirms that larger prey (rats, hamsters) remain mobile and survive longer – presumably traveling further before dying (Hayes, 1992a) – than smaller prey (mice). This result seems intuitive, but ontogenetic differences in rodent susceptibility has led to conflict regarding the effects of prey size on venom susceptibility (see Hayes, this volume). Although size differences undoubtedly influence survival, physiological differences are important as well, as hamsters survived longer than rats despite having equivalent mass. To minimize the risk of losing envenomated prey that might scamper beyond recovery range before dying, rattlesnakes should and do inject more venom into larger prey

(Hayes, 1995; Hayes et al., 1995, 2002). Second, the significant effect of dose and lack of an interaction between prey type and dose confirms that delivery of more venom hastens immobilization and death regardless of prey type. However, the optimal quantity of venom to inject remains unclear for the larger prey. The pairwise comparisons in experiment 2 lacked statistical power to identify a point of diminishing returns (optimas) for the rats and hamsters. Because of scaling issues (ratios of prey to snake size; Hayes, this volume), perceptual errors and bias (Hayes, this volume), and variable venom resistance of prey, rattlesnakes should not be expected to inject optimal quantities of venom into all prey types. In terms of prey size, we expect them to inject more venom into larger prey but not necessarily an optimal amount.

Ideally, adaptations are best demonstrated by an ensemble of optimality tests that support predictions of optimal function (Orzack and Sober, 2001). Our study here offers only tacit support for optimality, as we have focused on a single species, a single context (predation), and a single target type (rodents). A number of studies now suggest that snakes inject quantities of venom that fit expectations of adaptive function for other contexts and targets (Hayes, this volume), but several compelling issues warrant further discussion.

First, although we considered only time to immobilization and death, other functions of venom could also shape the optimal quantity of venom to inject in a predatory bite. Selection might favor different optimas for marking and relocating prey released after envenomation, or for accelerating digestion of prey (Hayes et al., 2002; Hayes, this volume). Because optimas undoubtedly vary among

different prey species (which vary in size and venom resistance), selection might also favor prey-specific strategies for venom expenditure (Hayes, 1992b; Hayes et al., 2002). In spite of these diverse and potentially competing influences on the optimal quantity of venom to deploy, we have documented good correspondence between actual venom delivery and the immediate, critical effects required to secure a preferred prey item (immobilization and death in adult mice).

Second, beyond the competing optimas hypothesized above, optimality may be constrained by a host of additional factors, including phylogenetic inertia, competing selection on other traits (e.g., physiology, venom toxicity) and other contexts (i.e., defensive use of venom), and environmental changes that affect both predator and prey populations. Ultimately, optimality for any one trait may not be achievable (e.g., Stephens and Krebs, 1986; Orzack and Sober, 2001). Even so, we expect selection to be strongest for those traits that are most critical for fitness differences among individuals. In the case of venom expenditure by rattlesnakes, we suspect that foraging success on a major dietary item is more critical than other uses of venom, as feeding must occur frequently and usually requires venom expenditure, foraging success can profoundly influence fitness (e.g., Taylor et al., 2005), and snakes often rely on strategies other than venom injection to defend themselves (e.g., Duvall et al., 1985; Gibbons and Dorcas, 2002; Glaudas, 2004; see other references in Hayes, this volume).

Third, we recognize that an optimal trait functions best relative to alternative traits and traits possessed by other individuals in the population

(Orzack and Sober, 2001). To more rigorously assess optimality in snake venom expenditure, future studies should develop and test mathematically explicit models and address phenotypic variation and the fitness differences associated with such variation.

Fourth, the question remains as to what exactly selection might act on to influence the quantities of venom expended. There are two obvious possibilities: the morphology and kinematics of the venom apparatus (i.e., physics) and decision-making by the snake (i.e., cognition). Young (this volume) contends that variation in venom expenditure by rattlesnakes is largely a consequence of physics, especially forces acting on the fang sheath. If venom expulsion is limited by physics, we suggest that venom delivery could approach optimality for only a narrow set of contexts or targets. To illustrate this, we assume that the venom apparatus of a given species is optimized for feeding on rodents. In this case, control of venom delivery by fang sheath kinematics could plausibly yield greater venom delivery into larger prey (thicker skin might cause greater fang sheath displacement and correspondingly more venom flow; Young et al., 2002, 2003; Young, this volume), which might be adaptive for a wide range of rodents consumed. However, if the snake also feeds infrequently on alternative prey, such as anurans, lizards, or invertebrates having very different skin properties, venom delivery would be more happenstance than adaptive (though other predatory tactics, such as holding on to prey, could help ensure feeding success, yet we would consider such tactics to be cognition). Further, an optimal quantity for defensive bites might be compromised if the system is optimized for predatory

bites. As an alternative to Young's hypothesis, we espouse the view that snakes possess cognitive control of venom expenditure and can make decisions about how much venom to inject (Hayes et al., 2002; Hayes, this volume). Accordingly, selection could favor cognitive strategies that optimize venom expenditure for a much wider range of contexts and targets. Cognitive control would allow the snake to compensate for situations in which optimal venom delivery is constrained by the physics of the venom delivery system.

Finally, to support our view of the importance of cognition, many animals other than snakes also expend venom quantities that vary depending on context and target (Hayes et al., 2002; Stewart and Gilly, 2005; Hostettler and Nentwig, 2006). Although neurologically much simpler than a snake, the spider *Cupiennius salei*, for example, recognizes how much venom is available in its glands and makes decisions about whether to attack, which species to attack, and how much venom to use (Wigger et al., 2002; Wullschleger and Nentwig, 2002; Kuhn-Nentwig et al., 2004; Hostettler and Nentwig, 2006). We should not be surprised that a snake might make such decisions as well (Hayes et al., this volume). The well-documented invertebrate examples, our previous studies of snakes, and the present study collectively support our contention that selection can act strongly on the quantities of venom expended by venomous animals.

LITERATURE CITED

Duvall, D., M. B. King, and K. J. Gutzwiller. 1985. Behavioral ecology and ethology of the prairie rattlesnake. Nat. Geo. Res. 6:457 469.

Duvall, D., M. J. Goode, W. K. Hayes, J. K. Leonhardt, and D. Brown. 1990. Prairie rattlesnake vernal migration: Field experimental analyses and survival value in spring. Nat. Geo. Res. 6:457-469.

Gibbons, J. W., and M. E. Dorcas. 2002. Defensive bite behavior of cottonmouths (*Agkistrodon piscivorus*) toward humans. Copeia 2002:195-198.

Glaudas, X. 2004. Do cottonmouths (*Agkistrodon piscivorus*) habituate to human confrontations? Southeast. Nat. 3:129-138.

Goodarzi, H., H. S. Najafabadi, K. Hassani, H. A. Nejad, and N. Torabi. 2005. On the optimality of the genetic code with the consideration of coevolution theory by comparison of prominent cost measure matrices. J. Theor. Biol. 235:318-325.

Hayes, W. K. 1991. Envenomation strategies of prairie rattlesnakes. Ph.D. Dissertation, University of Wyoming, Laramie.

Hayes, W. K. 1992a. Factors associated with the mass of venom expended by prairie rattlesnakes (*Crotalus v. viridis*) feeding on mice. Toxicon 30:449-460.

Hayes, W. K. 1992b. Prey-handling and envenomation strategies of prairie rattlesnakes (*Crotalus v. viridis*) feeding on mice and sparrows. J. Herpetol. 26:496-499.

Hayes, W. K. 1995. Venom metering by juvenile prairie rattlesnakes, *Crotalus v. viridis*: effects of prey size and experience. Anim. Behav. 50:33-40.

Hayes, W. K., I. I. Kaiser, and D. Duvall. 1992. The mass of venom expended by prairie rattlesnakes when feeding on rodent prey. Pp. 383-388 *in* J. A. Campbell and E. D. Brodie, Jr. (Eds.), Biology of the Pitvipers. Selva, Tyler, Texas.

Hayes, W. K., P. A. Lavin-Murcio, and K. V. Kardong. 1995. Northern Pacific rattlesnakes (*Crotalus viridis oreganus*) meter venom when feeding on prey of different sizes. Copeia 1995:337-343.

Hayes, W. K., S. S. Herbert, G. C. Rehling, and J. Gennaro. 2002. Factors that influence venom expenditure in viperids and other snake species during predatory and defensive contexts. Pp. 207-233 in G.W. Schuett, M. Hoggren, M. E. Douglas, and H.W. Greene (Eds.), Biology of the Vipers. Eagle Mountain Publ., Eagle Mountain, Utah.

Hostettler, S., and W. Nentwig. 2006. Olfactory information saves venom during prey-capture of the hunting spider *Cupiennius salei* (Araneae: Ctenidae). Funct. Ecol. 20:369-375.

Kardong, K. V. 1986. The strike behavior of the rattlesnake, *Crotalus viridis oreganus*. J. Comp. Psychol. 100:304-314.

Kardong, K. V. 2002. Colubrid snakes and Duvernoy's "venom" glands. Journal of Toxicology-Toxin Reviews 21:1-19.

Kardong, K. V., and T. L. Smith. 2002. Proximate factors involved in rattlesnake predatory behavior: a review. Pp. 253-266 in G.W. Schuett, M. Hoggren, M. E. Douglas, and H.W. Greene (Eds.), Biology of the Vipers. Eagle Mountain Publ., Eagle Mountain, Utah.

Klauber, L. 1936. A statistical study of the rattlesnakes. VI. Fangs. Occ. Pap. San Diego Soc. Nat. Hist. 5:1-61.

Kuhn-Nentwig, L., J. Schaller, and W. Nentwig. 2004. Biochemistry, toxicology and ecology of the venom of the spider *Cupiennius salei* (Ctenidae). Toxicon 43:543-553.

McCue, M.D. In press. Cost of producing venom in North American pitvipers. Copeia. Orzack, S., and E. Sober (Eds.). 2001. Adaptationism and Optimality. Cambridge Univ. Press, Cambridge.

Perry, G., and E. R. Pianka. 1997. Animal foraging: Past, present and future. Trends Ecol. Evol. 12:360-364.

Sells, P. G. 2003. Animal experimentation in snake venom research and in vitro alternatives. Toxicon 42:115-133.

Stephens, D. W., and J. R. Krebs. 1986. Foraging Theory. Princeton Univ. Press., Princeton, New Jersey.

Stewart, J., and W. F. Gilly. 2005. Piscivorous behavior of a temperate cone snail, *Conus californicus*. Biol. Bull. 209:146-153.

Taylor, E. N., M. A. Malawy, D. M. Browning, S. V. Lemar, and D. F. DeNardo. 2005. Effects of food supplementation on the physiological ecology of female western diamond-backed rattlesnakes (*Crotalus atrox*). Oecologia. 144(2): 206-213.

Thorington, G., and D. Hessinger. 1998. Efferent mechanisms of discharging Cnidae: II. A nematocyst release response in the sea anemone tentacle. Biol. Bull. 195:145-55.

Todorov, E. 2004. Optimality principles in sensorimotor control. Nat. Neurosci. 7:907-915.

Wigger, E., L. Kuhn-Nentwig, and W. Nentwig. 2002. The venom optimisation hypothesis: a spider injects large venom quantities only into difficult prey types. Toxicon 40:749-752.

Wullschleger, B., and W. Nentwig. 2002. Influence of venom availability on a spider's prey-choice behaviour. Funct. Ecol. 16:802-807.

Young, B. A., C. E. Lee, and K. M. Daley. 2002. Do snakes meter venom? Bioscience 52:1121-1126.

Young, B. A., M. Phelan, M. Morain, M. Ommundsen, and R. Kurt. 2003. Venom injection in rattlesnakes (*Crotalus*): peripheral resistance and the pressure-balance hypothesis. Can. J. Zool. 81:313-320.

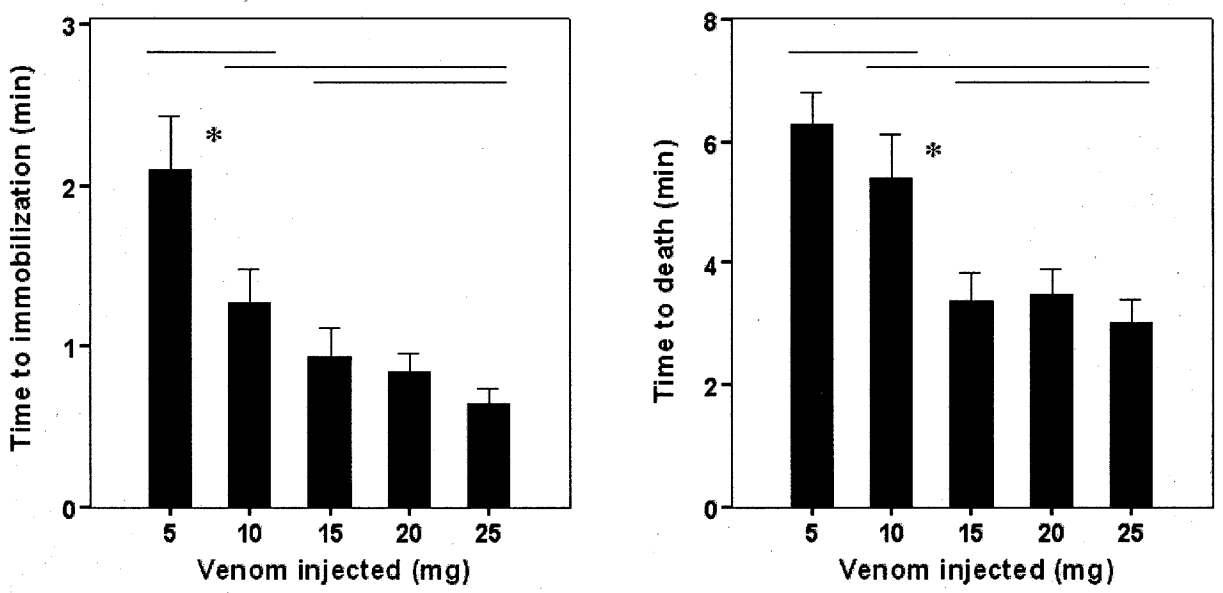


Figure 4-1. Mean (+ 1 S.E.) time to immobilization and death (min) for adult mice (*Mus musculus*) injected with varying doses of Prairie Rattlesnake (*Crotalus v. viridis*) venom. Horizontal lines represent equivalent groups identified by Tukey's multiple comparisons of rank-transformed data. Asterisks indicate significant pairwise differences (for adjacent doses) identified by Tukey's multiple comparisons of non-transformed data. Sample size for each mean ranged from 21-22.

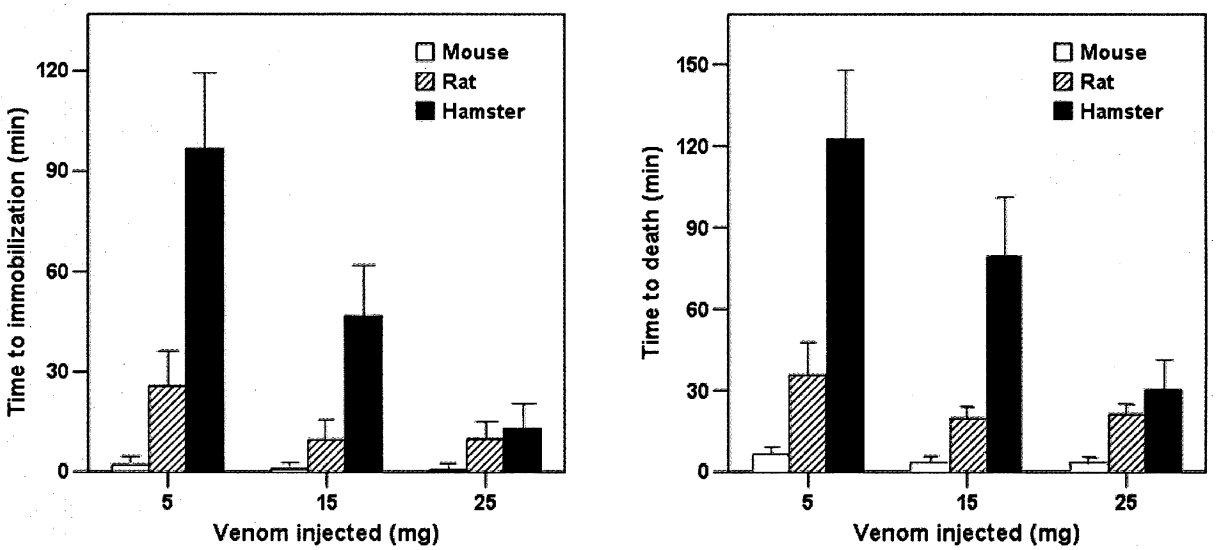


Figure 4-2. Mean (+ 1 S.E.) time to immobilization and death (min) for three prey species of varying size and venom resistance (mice, *Mus musculus*; rats, *Rattus norvegicus*; and hamsters, *Mesocricetus auratus*) injected with varying doses of Prairie Rattlesnake (*Crotalus v. viridis*) venom. Sample size for each of the three means (5, 15, and 25 mg, respectively) varied among the mice (N = 21, 22, 22), rats (N = 12 each), and hamsters (N = 6, 6, 9).

CHAPTER V

Denim Clothing Reduces Venom Expenditure by Southern Pacific Rattlesnakes (*Crotalus oreganus helleri*) During Defensive Bites at Model Human Limbs

Shelton S. Herbert and William K. Hayes

Department of Earth and Biological Sciences, Loma Linda University, Loma Linda, CA 92354

ABSTRACT

Venomous snakebites can be painful, costly, and potentially life-threatening. Because the severity of envenomation depends on the mass of venom injected during the bite, effective measures should be studied that can reduce the amount of venom injected. We experimentally evaluated the possibility that clothing (denim material from blue jeans) covering a model human limb (a warm, saline-filled glove) would interfere with the kinematics of venom delivery, thereby reducing the amount of venom injected into the model during defensive bites by the Southern Pacific Rattlesnake (*Crotalus oreganus helleri*), a representative viper. Denim-covered gloves received significantly less venom than bare gloves, with a 60% reduction in venom injected by small snakes and 66% by large snakes. Latency to bite, number of bites, and duration of fang contact were similar for the two glove types, suggesting that the two targets elicited similar defensive behaviors and strikes. Several findings suggested that denim interfered with venom delivery, including the proportion of venom spilled on the glove in relation to both snake size and total venom expended. Large

rattlesnakes struck more readily, maintained longer fang contact during the bite, and expended more venom than small snakes. We recommend that long pants be considered a simple, low-cost, and potentially effective means of providing a measure of protection from snakebite when in the habitat of venomous snakes.

INTRODUCTION

As human populations continue to expand and encroach upon the habitat of venomous snakes, encounters between humans and venomous snakes potentially ending in envenomation of the humans will persist (Whitaker et al., 2000; Whitaker and Shine, 1999; Chippaux, 1998). Recent studies suggest that more than 1 million venomous snakebites occur globally each year, resulting in as many as 100,000 deaths and countless more cases of long-term disability (e.g., Chippaux, 1998, 2006; Gutierrez et al., 2006).

The personal and financial costs of venomous bites can be substantial. The costs can include, but are not limited to: 1) transport to a hospital and often between hospitals (ambulance or helicopter; e.g., McKinney, 2001; Sharma et al., 2004; Chauhan et al., 2005); 2) emergency room treatment and hospitalization (e.g., Lopoo et al., 1998; Tanen et al., 2001; Cheng and Currie, 2004); 3) antivenom administration (e.g., Fry et al., 2003; Pizon et al., 2007); 4) surgical intervention, such as fasciotomy (Hall, 2001; Juckett and Hancox, 2002; Chattopadhyay et al., 2004); and 4) subsequent physical and/or occupational therapy. Additional costs borne by the patient or family include lost income from time off work or death (Spiller and Bosse, 2003; Sharma et al., 2004). Although

mortality is relatively rare, particularly in developed countries, morbidity can exact an extraordinary toll (e.g., Dart et al., 1992; Spiller and Bosse, 2003; Gutierrez et al., 2006).

Any practical solutions that might reduce the frequency or severity of snakebites warrant evaluation. Preventative measures should begin with alertness to one's surroundings and awareness of habitats and conditions that favor snake encounters (Whitaker and Shine, 1999). Appropriate (and inappropriate) first aid measures should be understood, with proficiency in applying the appropriate measures and avoiding those that are inappropriate (e.g., McKinney, 2001; Cheng and Currie, 2004; German et al., 2005; Rogers and Winkel, 2005). Protective footware or clothing also can be worn that protects against fang penetration (e.g., da Silva et al., 2003; Currie, 2004; Hon et al., 2004) or reduces the amount of venom injected. Indeed, the severity of the bite is due largely to the amount of venom injected into the person, which covaries with snake size (Hayes et al., 2002).

Numerous products are sold that purportedly protect against snakebite. These products include special penetration-resistant pants, chaps, gaiters, and boots. Generally, these products are worn primarily by snake specialists (e.g., Morandi and Williams, 1997) and their use can impede efficient movement through snake habitats. The efficacy of these products is seldom, if ever, tested and published.

Although ordinary clothing (e.g., long pants, long-sleeved shirts) is vulnerable to fang penetration (da Silva et al., 2003), we wondered whether

denim material—the material frequently used for long pants, or "blue jeans"—might reduce the severity of envenomation. A denim barrier may interfere with the kinematics of the bite by deflecting the fangs, disrupting jaw and fang movements, altering fang penetration depth and trajectories, and mistiming venom expulsion (Hayes et al., 2002; Hayes, 2007).

The purpose of this study was to test whether the presence of a denim covering provided significantly reduced the amount of venom injected during a defensive bite at a model human limb. In doing so, we also considered how the potential protective effect might vary with snake size.

METHODS

Snakes.-The viperid snakes used in this experiment were eight small (35-54 cm snout-vent length, SVL) and nine large (66-102 cm SVL) Southern Pacific Rattlesnake (*Crotalus oreganus helleri*). Snakes were individually maintained in assorted cage sizes with a light:dark cycle of 14:10 hours at 25-27 C. Each cage included pine shavings for substrate and a glass vessel containing water ad libidum. The snakes were fed laboratory mice (*Mus musculus*) every two weeks (13-15 d) and were fasted at this interval prior to each strike trial.

Conditions.-We prepared two conditions to elicit defensive bites. The first was a bare human limb model comprised of a heavy-duty latex glove filled with 500 mL of warm (38° C) phosphate-buffered saline (PBS) and secured with a plastic zip tie. The glove was also rubbed against the investigator's arms to transfer human scent. The second was identical, except that the glove was

covered with denim material. In both conditions, the model was suspended from an aluminum snake hook by an additional zip tie for presentation to the snake. The model was able to swing freely from the hook.

Strike trials.-Snakes were individually transferred by snake hook to a 1 x 1 x 0.6 m (L x W x H) wooden arena with a fresh 1 x 1 m craft paper floor covering and allowed 5 min for acclimation. The arena was lighted from above by three 100-W bulbs within metal reflectors approximately 1.25 m above the floor. Each snake was tested twice, once with the bare glove and once with the denim-covered glove. The sequence of presentation was randomized such that half the snakes were assigned the bare glove first and half were assigned the denim-covered glove first. Trials were recorded by an S-VHS camcorder (Panasonic PV-S7700-A) at standard tape speed (30 fields/sec) with a 1/500 sec shutter speed. The camera was positioned at approximately 1.25 m obliquely above the arena.

Presentation of the glove was standardized to consist of approximately 5 sec of non-contact harassment followed by a thrust of the model toward the snake (but avoiding contact). This sequence was repeated until a bite occurred or until 15 min elapsed, at which point the trial was terminated. On some occasions, the snake managed to bite twice the glove twice before we could retrieve it. In all trials, the snake behaviors and strikes elicited were unambiguously defensive, accompanied by considerable rattling, head-elevated body coiling, and prolonged arcing tongue-flicks interrupted by occasional escape crawling (Hayes and Duvall, 1991). Immediately following a bite, the

glove was immediately transferred to a clean 1 L beaker, whereupon the denim cover was removed if present and placed within a plastic zip-lock bag. The PBS-filled glove from was then gently mixed (rocked back and forth) to ensure even distribution of the venom and then dumped into the beaker for further mixing before transferring a 10-mL sample by plastic transfer pipette into a plastic test tube. Occasional fluid spillage through fang punctures in the gloves was deemed a trivial source of venom loss. The denim covers were then placed in 400 mL PBS and agitated for 2 min before transferring another 10-mL sample into a plastic test tube. Both the glove and denim cover samples were frozen at -20 C for subsequent venom assays.

Venom measurements.–A total protein assay (Coomassie 1-25 µg/mL protocol, Pierce Chemical Co.) was used to quantify venom in the experimental samples. Accomplishing this required appropriate control samples to derive standard curves.

Control standards for the glove samples were created by injecting seven PBS-filled bare gloves with different quantities of *C. atrox* venom (0, 20, 40, 60, 80, and 100 mg dissolved in 0.5 mL phosphate-buffered saline at pH 7.4; purchased from Kentucky Reptile Zoo, Slade, Kentucky) using a tuberculin syringe and 22-ga needle. These control gloves and samples derived from them were treated in a manner identical to the experimental gloves, including handling with bare hands.

Control standards for the denim covers were created from six clean denim covers, each rinsed in 1 L deionized water for 5 min and then allowed to dry. The

denim covers were placed in a beaker and then injected by syringe and needle with 0, 2, 4, 8, 10, and 12 mg *C. atrox* venom dissolved in 0.5 mL PBS. The denim covers were then treated in a manner identical to the experimental gloves.

The experimental and control glove samples were assayed together, in triplicate, on 96-well microtitre plates (Corning, cat. # 430247). The experimental and control denim cover samples were likewise assayed together, in triplicate, on plates separate from the glove samples. Absorbance values (570 nm) from the controls were used to generate separate standard curves for the glove and denim cover samples. The standard curves were used to estimate the mass of venom (mg) injected by snakes using linear regression equation. When absorbance values from experimental samples exceeded those from the standard curve, the experimental sample was diluted up to ten-fold and assayed again. Calculations for venom mass of diluted samples were adjusted to reflect their original concentration. The coefficients of determination for the standard curves were indicative of the high reliability of the assays (all $r^2 \geq 0.88$).

Dependent measures.–During frame-by-frame videotape review, we recorded for each strike trial the latency to bite, number of bites, and duration of fang contact with the model (defensive strikes by rattlesnakes almost always involve a quick bite and release). In some cases, incomplete video records (camcorder not turned on when glove was introduced to the arena, or the glove obscured the snake's biting actions from the camera) reduced the sample size for behavioral variables. From the protein assays, we determined the mass of venom expended (nearest milligram, dry mass) in each glove model and denim

cover. For models covered with denim, we computed the proportion of venom spilled on the denim (venom spilled on the denim divided by sum of venom injected into glove and venom spilled on the denim). Because our primary interests were in whether denim reduced the amount of venom injected into the target, we did not adjust the mass of venom expended for the few targets that received multiple bites (c.f., Hayes, 1992).

Data analyses.–All data were analyzed using SPSS 13.0 for Windows. To meet parametric assumptions, duration of fang contact, all measures of venom expended, and proportion of venom spilled on the denim were rank-transformed. Most statistical tests involved 2 x 2 mixed analyses of variance (ANOVAs), for which glove condition (bare, denim-covered) was treated as a within-subjects factor and snake size (small, large) as a between-subjects factor. We also relied on two-tailed *t*-tests, Pearson's correlation analyses, and a non-parametric McNemar test. For the ANOVAs, effect sizes indicating the approximate proportion of variance explained by a dependent variable or interaction were computed as partial eta-square (η^2) values. When the partial η^2 values for main effects and interactions exceeded 1.0, we adjusted these by dividing each partial η^2 by the sum of all partial η^2 values. Alpha levels of 0.05 were used for all tests.

RESULTS

A total of 31 bites were obtained from the 17 snakes. However, sample sizes for most statistical tests were limited to the 5 small and 7 large snakes that had complete venom data for both glove conditions. For most dependent

variables, comparisons between the two size classes and two glove conditions can be seen in Table 5-1.

Latency to bite.-The ANOVA revealed no differences in duration of harassment before biting between the two glove conditions ($F_{1,8}$= 0.07, P = 0.80, partial η^2 = 0.01) and between the two snake size classes ($F_{1,8}$ = 4.53, P = 0.066, partial η^2 = 0.36). However, the effect size for snake size class was substantial, suggesting that large snakes probably struck more quickly than small snakes (means = 1.3 and 3.7 min, respectively, when pooled for both glove conditions; see Table 4-1). There was no interaction between glove condition and snake size ($F_{1,8}$ = 0.12, P = 0.74, partial η^2 = 0.06). Accordingly, both gloves elicited similar responsiveness from the snakes.

Number of bites.-The majority of trials involved single defensive bites. However, two rapid bites occurred in one (6.7%) of the 15 trials involving bites by small snakes and three (18.8%) of the 16 trials involving bites by large snakes. Because of pseudoreplication (most but not all snakes were observed biting in each of two conditions), these data were not suitable for statistical evaluation. After pooling bites by small and large snakes for those having complete data, a McNemar's test revealed no significant difference in the proportion of trials involving multiple bites between bare (8.3% of 12 trials) and denim-covered (25% of 12 trials) human limb models (exact two-tailed P = 0.63).

Contact duration.-Mean values were highly inflated by three bites involving difficulty with fang disengagement, thus increasing fang contact duration to 2.63 - 7.29 sec, well over the typical 0.20-0.25 sec for strikes at gloves by large

rattlesnakes (Hayes et al., 2002). One bite of the bare glove required 5.33 sec and two bites of the denim-covered gloves required 2.63 and 7.29 sec for disengagement; all other bites involved ≤0.33 sec. Accordingly, rank-transformed data were used for statistical analysis and median values are reported with the means in Table 4-1. The ANOVA yielded no differences between the two glove conditions ($F_{1,7}$ = 1.74, P = 0.23, partial η^2 = 0.20), though the effect size was moderate. However, the significant difference for size class ($F_{1,7}$ = 6.89, P = 0.03, partial η^2 = 0.50) indicated that large snakes maintained longer fang contact than the small snakes (median = 0.20 and 0.13 sec, respectively, when pooled for both glove conditions; see Table 4-1). There was no interaction between glove condition and size class ($F_{1,7}$ = 0.02, P = 0.90, partial η^2 < 0.01).

Venom expended.-The first ANOVA examined how much venom was injected into the gloves (Table 4-1), which should correspond to venom injected into human tissues. The significant effect of glove condition confirmed that snakes injected approximately two-thirds less venom into the denim-covered glove than into the bare glove (small snakes before rounding to nearest 1 mg: 60% less; large snakes: 66% less; $F_{1,10}$ = 6.47, P = 0.029, adjusted partial η^2 = 0.35). Snake size was also significant, with large snakes injecting twice as much venom as the small snakes ($F_{1,10}$ = 14.86, P = 0.003, adjusted partial η^2 = 0.54). There was no interaction between these variables ($F_{1,10}$ = 1.39, P = 0.027, adjusted partial η^2 = 0.11), suggesting that glove interference with venom injection was similar for the two size classes.

The second ANOVA considered total venom expended by the snakes, including that spilled harmlessly in the denim covers (Table 4-1). There was a significant interaction between glove condition and snake size ($F_{1,10}$ = 12.77, P = 0.005, adjusted η^2 = 0.40), suggesting that the difference between the two glove conditions depended on snake size. Paired t-tests for the simple main effects of glove condition indicated that small snakes expended significantly more venom when biting denim-covered gloves compared to bare gloves (t = 4.14, df = 4, P = 0.014), whereas large snakes expended similar amounts of venom for the two glove conditions (t = 1.86, df = 6, P = 0.112). Regardless of glove condition, large snakes expended more venom than the small snakes ($F_{1,10}$ = 38.64, P< 0.001, adjusted partial η^2 = 0.56).

For bites at denim-covered gloves, small snakes spilled 8.6 mg of venom on the denim (87% of total venom expenditure), whereas large snakes spilled 21 mg (55% of total venom expenditure). In spite of the 32% (1 S.E. = 0.18) difference, an independent-samples t-test showed that the proportion of venom spilled was similar for the two snake size classes (t = 1.142, df = 10, P = 0.280). Finally, venom spilled on the denim was negatively correlated with the total amount of venom expended (all snakes pooled: r^2 = 0.66, P < 0.001).

DISCUSSION

From the perspective of a potential snakebite victim, our results suggest that wearing long pants (e.g., blue jeans) when in snake habitat can substantially reduce the amount of venom a snake injects during a defensive bite. The

reduction in venom injected into denim-covered model human limbs was approximately two-thirds for both small and large rattlesnakes. As a consequence, the severity of envenomation for human snakebite victims could be substantially reduced, on average, by wearing clothing that covers the limbs. Even so, high variability in venom expenditure by snakes during defensive bites must be anticipated (Hayes et al., 2002), and substantial envenomation through clothing can still occur (S. P. Bush, pers. comm.). Although we studied a single representative viper species, we anticipate that clothing should reduce venom injection for most venomous snake species.

What caused the reduction in venom injected into the model human limbs? Two possibilities could be considered: either the snakes perceived and responded to the two targets differently, or the denim covering interfered with venom delivery.

Several findings in our study suggest that the snakes responded similarly to the two targets. First, the two targets presented different visual-thermal images to the snakes, which might have affected the snakes' perception of and defensive responses to the threat. However, the two conditions elicited similar behaviors from the snakes in terms of latency to strike, number of bites delivered, and duration of fang contact. Thus, the differences should not have resulted from target features overtly affecting prestrike behaviors, the tendency to launch strikes, or bite duration, the latter being a key kinematic variable that affects venom delivery during defensive bites (Herbert, 1998; Hayes et al., 2002; 2007).

Other results support our interpretation that clothing interferes with venom delivery. First, there was a substantial difference in the quantity of venom injected into the two glove types. Glove type explained approximately 35% of the variation in venom injected into the gloves. Second, more venom was delivered into the bare gloves during a period of fang contact equal to that of the denim-covered gloves. We suspect this resulted from a greater period of fang contact with the glove itself (as opposed to the denim clothing), allowing more time for venom to be injected into the glove. Third, a negative correlation existed between venom spillage on the denim and total venom expended. Bites presumably disrupted by the denim, resulting in considerable venom spilled onto the denim, somehow reduced the total amount of venom the snakes expulsed. Conversely, when fangs appeared to cleanly penetrate the denim, the snakes were able to eject a larger bolus of venom. The difference between these two scenarios could reflect kinematic constraints and/or venom metering (decision-making) by the snakes (Hayes, 2007). Finally, we expected the small snakes, with shorter fangs, to be less efficient penetrating the denim material and delivering venom into the gloves. Although the we were unable to document such a difference, as the proportion of venom spilled on the denim was statistically similar for the small (87%) and large (55%) snakes, we suspect that a larger sample size might have revealed such a difference.

In terms of total venom expended by the snakes, there was an unexpected interaction between snake size and glove type. Although the large snakes ejected similar quantities of total venom into the two gloves, the small

snakes expended more venom when biting the denim-covered gloves. This difference could result from two possibilities. First, the small snakes may have responded more so than large snakes to the different target properties by attempting to inject more venom when denim was present. Alternatively, the denim covers on the human limb models presumably picked up extraneous proteins from the strike arena substrate (Rehling, 2002). The ratio of extraneous protein to venom expended was likely trivial for the large snakes, but may well have added significantly to the total venom protein measured for the small snakes. We favor the latter explanation.

Despite popular beliefs, a growing body of evidence clearly indicates that large venomous snakes, including rattlesnakes, are much more dangerous to humans than small ones. In some venomous species, large snakes may strike more readily (Whitaker et al., 2000), as supported by the large effect size for time to strike in the present study, but this may not be characteristic of all taxa or defensive contexts (Shine et al., 2002). Large snakes may strike with greater velocity, distance, and/or accuracy (Rowe and Owings, 1990; Whitaker et al., 2000; Shine et al., 2002). Large snakes may maintain longer fang contact with the target during the bite (Rowe and Owings, 1990), as supported by the difference observed in the present study (but see Herbert, 1998). Larger snakes also inject substantially more venom than smaller snakes (Hayes 1992, 2007; Herbert, 1998; Hayes et al., 2002), as supported once again by the present findings. Greater venom expenditure by large snakes results from more venom available and the greater rates of venom flow through larger-diameter ducts and

fangs (Herbert, 1998; Hayes et al., 2002, in press). Importantly, our findings here demonstrate that clothing worn over the limbs can reduce venom expenditure by small and large snakes alike.

LITERATURE CITED

Dart, R. C., J. T. McNally, D. W. Spaite, and R. Gustafson. 1992. The sequelae of pitviper poisoning in the United States. Pp. 395-404 *in* J. A. Campbell and E. D. Brodie, Jr., eds. Biology of the Pitvipers. Selva, Tyler, Texas.

da Silva, C. J., M. T. Jorge and L. A. Ribeiro. 2003. Epidemiology of snakebites in a central region of Brazil. Toxicon 41:251-255.

Chattopadhyay, A., R. D. Patra, V. Shenoy, V. Kumar, and Y. Nagendhar. 2004. Surgical implications of snakebites. Indian Journal of Pediatrics 71:397-399.

Chippaux, J. P. and M. Goyffon. 2006. Venoms and poisonous animals – I. Overview. Med. Trop. (Mars). 66:215-220.

Chippaux, J. P. 1998. Snakebites: appraisal of the global situation. Bull. World Health Org. 76:515-524

Chippaux, J. P. 2006. Snake Venoms and Envenomations. Krieger Publishing Company, Melbourne, Florida.

Currie, B. J. 2004. Snakebite in tropical Australia: a prospective study in the "Top End" of the Northern Territory. Med. J. Aust. 181:693-697.

Fry, B. G., K. D. Winkel, J. C. Wickramaratna, W. C. Hodgson, and W. Wuster. 2003. Effectiveness of snake antivenom: Species and regional venom variation and its clinical impact. Journal of Toxicology 22:23-34.

German, B. T., J. B. Hack, K. Brewer, and W. J. Meggs. 2005. Pressure-immobilization bandages delay toxicity in a porcine model of eastern coral snake (*Micrurus fulvius fulvius*) envenomation. Annals of Emergency Medicine 45:603-608.

Gutierrez, J. M., R. D. G. Theakston and D. Warrell. 2006. Confronting the neglected problem of snake bite: the need for a global partnership. PLoS Medicine Med 3:727-731.

Hall, E. L. 2001. Role of surgical intervention in the management of crotaline snake envenomation. Ann. Emerg. Med. 37:175-180.

Hayes, W. K. 1992. Factors associated with the mass of venom expended by prairie rattlesnakes (*Crotalus v. viridis*) feeding on mice. Toxicon 30:449-460.

Hayes, W. K. 2007. The snake venom-metering controversy: levels of analysis, assumptions, and evidence. In press *in* W. K. Hayes, K. R. Beaman, M. D. Cardwell, and S. P. Bush (eds.), The Biology of Rattlesnakes. Loma Linda University Press, Loma Linda, California.

Hayes, W. K., and D. Duvall. 1991. A field study of prairie rattlesnake predatory strikes. Herpetologica 47:78-81

Hayes, W. K., S. S. Herbert, G. C. Rehling, and J. Gennaro. 2002. Factors that influence venom expenditure in viperids and other snake species during predatory and defensive contexts. Pp. 207-233 *in* G.W. Schuett, M. Hoggren, M. E. Douglas, and H.W. Greene (eds.), Biology of the Vipers. Eagle Mountain Publ., Eagle Mountain, Utah.

Hayes, W. K., S. S. Herbert, J. Harrison and K. Wiley. In press. Venom metering during spitting versus biting: differential venom gland contraction regulates venom expenditure in the spitting cobra *Naja nigricollis*. J. Herpetol.

Herbert, S. S. 1998. Factors influencing venom expenditure during defensive bites by cottonmouths (*Agkistrodon piscivorus*) and rattlesnakes (*Crotalus viridis*, *Crotalus atrox*). M.S. Thesis, Loma Linda University, Loma Linda, California.

Hon, K. L., L. W. Kwok, and T. F. Leung. 2004. Snakebites in children in the densely populated city of Hong Kong: a 10-year survey. Acta Paediatrica 93:270-272.

Juckett, J., and J. Hancox. 2002. Venomous snakebites in the United States: management, review and update. Am. Fam. Phys. 65:1367-1378.

Lopoo, J. B., J. F. Bealer, P. C. Mantor, and D. W. Tuggle. 1998. Treating snakebitten children in North America: a study of pit viper bites. J. Ped. Surg. 33:1593-1595.

McKinney, P. E. 2001. Out-of-hospital and interhospital management of crotaline snakebite. Annals of Emergency Medicine 37:168-174.

Morandi, N., and J. Williams. 1997. Snakebite injuries: contributing factors and intentionality of exposure. Wilderness and Environmental Medicine 8:152-155.

Pizon, A. F., B. D. Riley, F. LoVecchio, and R. Gill. 2007. Safety and efficacy of Crotalidae Polyvalent Immune Fab in pediatric crotaline envenomations. Acad. Emerg. Med. 14:373-376.

Rehling, G. C. 2002. Venom expenditure in multiple bites by rattlesnakes and cottonmouths. M.S. Thesis, Loma Linda University, Loma Linda, California.

Rogers, I. R., and K. D. Winkel. 2005. Struan Sutherland's "rationalization of first-aid measures for elapid snakebite" - A commentary. Wild. Environ. Med. 16:160-163.

Rowe, M. P., and D. H. Owings. 1990. Probing, assessment, and management during interactions between ground squirrels and rattlesnakes. Part 1: risks related to rattlesnake size and body temperature. Ethology 86:237-249.

Sharma, S. K., F. Chappuis, N. Jha, P. A. Bovier, L. Loutan, and S. Koirala. 2004. Impact of snake bites and determinants of fatal outcomes in southeastern Nepal. Am J Trop Med Hyg. 71:234-238.

Shine, R., L. Sun, M. Fitzgerald, and M. Kearney. 2002. Antipredator responses of free-ranging pit vipers (*Gloydius shedaoensis*, Viperidae). Copeia 2002:843-850.

Spiller, H. A., and G. M. Bosse. 2003. Prospective study of morbidity associated with snakebite envenomations. Clin. Toxicol. 125-130.

Tanen, D., A. Ruha, K. Graeme, and S. Curry. 2001. Epidemiology and hospital course of rattlesnake envenomations cared for at a tertiary referral center in central Arizona. Acad. Emerg. Med. 8:177-182.

Whitaker, P. B., K. Ellis, and R. Shine. 2000. The defensive strike of the eastern brownsnake, *Pseudonaja textilis* (Elapidae). Funct. Ecol. 14:25-31.

Whitaker, P. B., and R. Shine. 1999. Responses of free-ranging brownsnakes (*Pseudonaja textilis*, Elapidae) to encounters with humans. Wildlife Res. 26:689-704.

Table 5-1. Mean (± 1 SE) values for variables associated with defensive bites by small and large Southern Pacific Rattlesnakes (*Crotalus oreganus helleri*). The targets were warm, saline-filled gloves (model human limbs) that were either bare or covered by denim clothing. Median values (for fang contact) and sample sizes (N) are within parentheses.

	Bare Glove		Denim Glove		Denim Cover		Denim Total (Glove + Cover)	
Dependent Measures	**Small**	**Large**	**Small**	**Large**	**Small**	**Large**	**Small**	**Large**
Latency to bite (min)	3.6 ± 1.2 (N = 5)	1.5 ± 0.6 (N = 5)	3.7 ± 1.3 (N = 5)	1.0 ± 0.6 (N = 5)	---	---	---	---
Fang contact duration (sec)	0.12 ± 0.03 (0.10; N = 4)	1.19 ± 1.03 (0.18; N = 4)	0.18 ± 0.05 (0.18; N = 5)	1.81 ± 1.17 (0.33; N = 5)	---	---	---	---
Venom expended (mg)	4 ± 1.5 (N = 5)	164 ± 42.6 (N = 7)	2 ± 0.9 (N = 5)	56 ± 25.2 (N = 7)	9 ± 0.4 (N = 5)	22 ± 7.4 (N = 7)	10 ± 1.0 (N = 5)	78 ± 31.0 (N = 7)

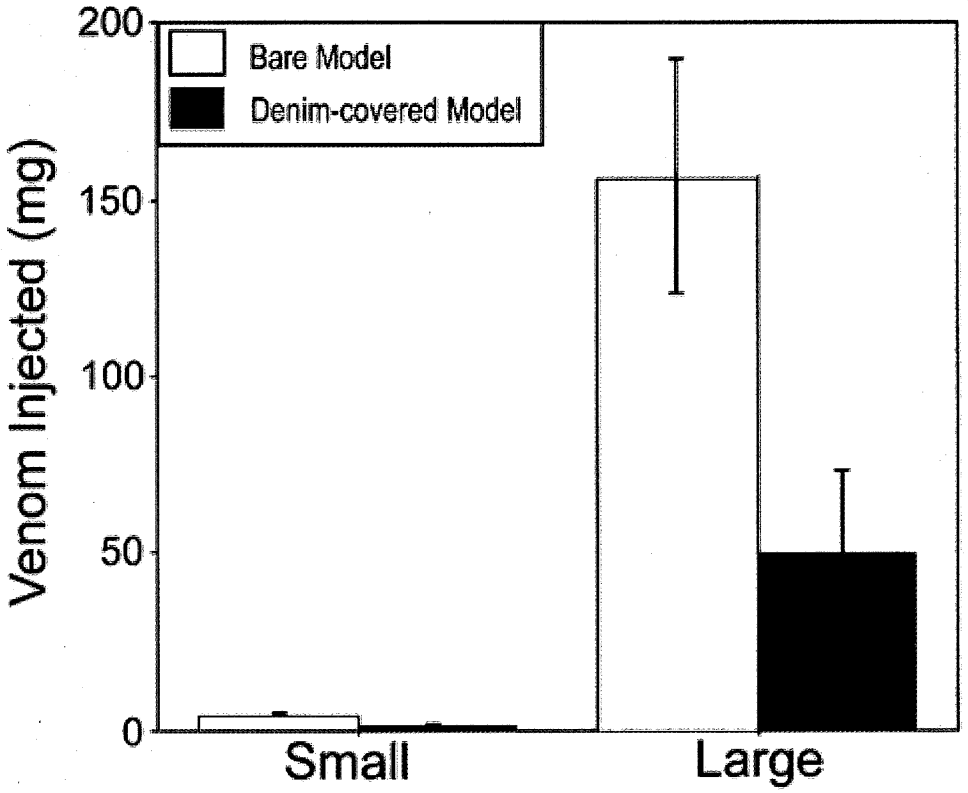


Figure 5-1. Mean (±1 S.E.) mass of venom injected into glove of bare and denim-covered human limb models by small (<40 cm) and large (>50 cm) Southern Pacific Rattlesnakes (*Crotalus oreganus helleri*).

CHAPTER VI

CONCLUSIONS ON VENOM EXPENDITURE BY SNAKES

In this dissertation, I examined some of the factors that influence venom expenditure by viperid and elapid snakes in both predatory and defensive contexts. I also considered the consequences of venom delivery into human snakebite victims. Here, I touch on some of the primary conclusions of each study.

Chapter II.— Our primary purpose in this study was to evaluate whether a representative spitting cobra, *N. n. nigricollis*, expends different quantities of venom during spitting and biting by means of differential venom gland contraction. Compared with spits, bites had nearly a four-fold ($P < 0.001$) increase in duration of venom flow and approximately a two-fold ($P = 0.031$) increase in the mass of venom expended.

Because venom gland contraction provided the only propulsive force for the venom expulsion (Young et al., 2002; Young, in press), our results confirm that *N. n. nigricollis* meters different quantities of venom during spitting and biting by means of differential venom gland contraction. Spits involve very brief contraction, whereas bites almost always involve lengthy contraction. Although not considered here, differences in the force of venom gland contraction are also possible.

Our data supported the view that the duration of venom flow corresponds to the quantity of venom expended. Based on effect sizes obtained from the ANCOVA model, this relationship (partial $\eta^2 = 0.25$) may be as strong as that between snake size and venom expenditure (partial $\eta^2 = 0.23$), which is well documented in a number of snake species (Hayes et al., 2002; Hayes, in press; Herbert, 1998).

Given the high degree of functional convergence, we see no *a priori* reason why viperids, like spitting cobras, could not similarly control duration (or possibly force) of venom gland contraction. Indeed, our analyses of venom flow duration support this view (Herbert, 1998; Hayes, in press). Although control of venom gland contraction may be important, venom metering can occur through other mechanisms (Hayes, in press) such as the duration of fang contact or the number of bites delivered.

Chapter III.—This study examined the effect of threat intensity on defensive bites by venomous snakes. We found that several behaviors associated with striking, including the quantity of venom expended, differed among the three levels of threat tested. Snakes were more likely to bite and did so more quickly at higher levels of threat, which would be consistent with risk assessment.

In the context of venom metering, the most important finding was that snakes delivered different quantities of venom depending on level of threat. Venom expenditure was statistically similar for bites in the low- and medium-threat conditions, which were elicited from unrestrained snakes by model human

limbs (saline-filled gloves). However, the snakes injected substantially more venom in the high-threat condition, when they were physically grasped by the investigator and presented a target (membrane-covered beaker) to bite voluntarily. Like other authors, we considered the latter condition to be one of last resort for the snake. At this point, any costs associated with use and replenishment of venom (McCue, 2006) might be outweighed by the benefit of inducing a painful, debilitating bite with maximum venom injection.

Collectively, the evidence suggests that snakes assess risk and modulate their behaviors, including venom expenditure, accordingly. Moreover, the analyses of venom expulsion suggest that differences in venom expenditure result from variation in duration of venom flow, presumably regulated by venom gland contraction and under central nervous system control of the snake.

Chapter IV.—The results of this study, particularly those of experiment 1 involving mice, support the view that adult Prairie Rattlesnakes expend a near-optimal quantity of venom when procuring adult rodent prey. Such an optimum represents a balance between energy expended (including venom synthesis and storage and relocation of dispatched prey) and energy procured from the prey. Delivery of more than 15 mg provided diminishing returns in terms of time to immobilization and death. The 16 mg predatory venom dose given by adult Prairie Rattlesnakes to mice appears to be close to an optimal (Hayes, 1992a).

The results of experiment 2 are more difficult to interpret because of the smaller sample sizes and correspondingly reduced statistical power. However, the significant effects of prey type (for both immobilization and death) confirms

that larger prey (rats, hamsters) remain mobile and survive longer – presumably traveling further before dying (Hayes, 1992a) – than smaller prey (mice). Although size differences undoubtedly influence survival, physiological differences are important as well, as hamsters survived longer than rats despite having equivalent mass. To minimize the risk of losing envenomated prey that might scamper beyond recovery range before dying, rattlesnakes should and do inject more venom into larger prey (Hayes, 1995; Hayes et al., 1995, 2002). The significant effect of dose and lack of an interaction between prey type and dose confirms that delivery of more venom hastens immobilization and death regardless of prey type. However, the optimal quantity of venom to inject remains unclear for the larger prey.

Although we considered time to immobilization and death, natural selection may also act on other functions of venom help shape the optimal quantity of venom to inject in a predatory bite. Selection might favor different optimas for marking and relocating envenomated prey, or for accelerating digestion of prey (Hayes et al., 2002; Hayes, 2007). Because optimas undoubtedly vary among different prey species (which vary in size and venom resistance), selection might also favor prey-specific strategies for venom expenditure (Hayes et al., 2002). In spite of these diverse and potentially competing influences on the optimal quantity of venom to deploy, we have documented good correspondence between actual venom delivery and the immediate, critical effects required to secure a preferred prey item (immobilization and death in adult mice).

Ultimately, optimality for any one trait may not be achievable (e.g., Stephens and Krebs, 1986; Orzack and Sober, 2001). Even so, we expect selection to be strongest for those traits that are most critical for fitness differences among individuals.

The question remains as to what exactly selection might act on to influence the quantities of venom expended. There are two obvious possibilities: the morphology and kinematics of the venom apparatus (i.e., physics) and decision-making by the snake (i.e., cognition). We espouse the view that snakes possess cognitive control of venom expenditure and can make decisions about how much venom to inject (Hayes et al., 2002; Hayes, 2007). Accordingly, selection could favor cognitive strategies that optimize venom expenditure for a much wider range of contexts and targets.

The evidence in this study for cognitive control corresponds well with many animals known to expend varying quantities of venom depending on context and target (Hayes et al., 2002; Stewart and Gilly, 2005; Hostettler and Nentwig, 2006). Such examples include spiders (*Cupiennius salei*). The well-documented invertebrate examples, our previous studies of snakes, and the present study collectively support our contention that selection can act strongly on the quantities of venom expended by venomous animals.

Chapter V.—From the perspective of a potential snakebite victim, our results suggest that wearing long pants (e.g., blue jeans) when in snake habitat can substantially reduce the amount of venom a snake injects during a defensive bite. The reduction in venom injected into denim-covered model human limbs

was approximately two-thirds for both small and large rattlesnakes. As a consequence, the severity of envenomation for human snakebite victims could be substantially reduced, on average, by wearing clothing that covers the limbs.

Our view is supported by our results. First, the snakes injected considerably more venom into bare models than into denim-covered models. Second, the greater venom mass injected into the bare glove occurred despite similar durations of fang contact. Third, the negative correlation between venom spillage on the denim and total venom expended suggests that bites presumably disrupted by the denim, resulted in considerable venom spilled onto the denim.

Despite popular beliefs, a growing body of evidence clearly indicates that large venomous snakes, including rattlesnakes, are much more dangerous to humans than small ones. Greater velocity, distance, and/or strike accuracy (Rowe and Owings, 1992; Whitaker et al., 2000; Shine et al., 2002), longer fang contact with the target during the bite (Rowe and Owings, 1990; Herbert, 1998; present study), and greater venom expenditure show that large snakes are much more dangerous to humans than smaller snakes (Hayes 1992b, 2007; Herbert, 1998; Hayes et al., 2002). Importantly, our findings here demonstrate that clothing worn over the limbs can reduce venom expenditure by small and large snakes alike.

REFERENCES

Aird, S. D. 2002. Ophidian envenomation strategies and the role of purines. Toxicon 40:335-393.

Chippaux, J. P. 1998. Snakebites: appraisal of the global situation. Bull. World Health Org. 76(5): pp 515-524

Chippaux, J. P. 2006. Snake Venoms and Envenomations. Krieger Publishing Company, Melbourne, Florida.

Chippaux, J. P., V. Williams, and J. White. 1991. Snake venom variability: methods of study, results and interpretation. Toxicon 29:1279-1303.

Chiszar, D., C. W. Radcliffe, and H. M. Smith 1992. Searching behaviors by rattlesnakes following predatory strikes. Pp. 369-382 *in* J. A. Campbell and E. D. Brodie, Jr, eds. Biology of the Pitvipers. Selva Selva, Tyler, Texas.

Chiszar, D., A. Walters, J. Urbaniak, H. M. Smith, and S. P. Mackessy. 1999. Discrimination between envenomated and nonenvenomated prey by western diamondback rattlesnakes (*Crotalus atrox*): chemosensory consequences of venom. Copeia 1999:640-648.

Coss, R. G., K. L. Guse, N. S. Poran, and D. G. Smith. 1993. Development of antisnake defenses in California ground squirrels (*Spermophilus beecheyi*): II. Microevolutionary effects of relaxed selection from rattlesnakes. Behaviour 124:137-164.

Dart, R. C., J. T. McNally, D. W. Spaite, and R. Gustafson. 1992. The sequelae of pitviper poisoning in the United States. Pp. 395-404 *in* J. A. Campbell and E. D. Brodie, Jr., eds. Biology of the Pitvipers. Selva, Tyler, Texas.

Fry, B. G. 2005. From genome to "venome:" molecular origin and evolution of the snake venom proteome inferred from phylogenetic analysis of toxin sequences and related body proteins. Genome Research 15:403-420.

Fry, B. G., and W. Wuster. 2004. Assembling an arsenal: origin and evolution of the snake venom proteome inferred from phylogenetic analysis of toxin sequences. Mol. Biol. Evol. 21:870-883.

Fry B. G., N. Vidal, J. A. Norman, F. J. Vonk, H. Scheib, R. Ramjan, S. Kuruppu, K. Fung, S. B. Hedges SB, M. K. Richardson, W. C. Hodgson, V. Ignjatovic, R. Summerhayes and E. Kochva E. 2005. Early evolution of the venom system in lizards and snakes. Nature 439:584-588.

Fry, B. G., H. Scheib, L. van der Weerd, B. Young, J. McNaughtan, S.F. Ryan Ramjan, N. Vidal, R.E., Poelmann, J.A. Norman. In press. Evolution of an arsenal: structural and functional diversification of the venom system in the advanced snakes (Caenophidia) in Molecular and Cellular Proteomics.

Gans, C. 1961. The feeding mechanism of snakes and its possible evolution. Am. Zool. 1:217-227.

Gutierrez, J. M. 2002. Understanding snake venoms: 50 years of research in Latin America. Rev. Biol. Trop. 50:377-394.

Gutierrez, J. M., R. D. G. Theakston and D. Warrell. 2006. Confronting the neglected problem of snake bite: the need for a global partnership. PLoS Medicine Med 3:727-731.

Haas, G. 1973. Muscles of the jaw and associated structures in the Rhyncocephalia and Squamata. Pp. 285-490 *in* C. Gans and T. Parsons, eds. Biology of the Reptilia, Vol. 4. Academic Press, New York.

Hardy, D. L. 1991. Epidemiology and medical management of rattlesnake bite in southern Arizona. Sonoran Herpetol. 4:153-158.

Hayes, W. K. 1992a. Factors associated with the mass of venom expended by prairie rattlesnakes (*Crotalus v. viridis*) feeding on mice. Toxicon 30:449-460.

Hayes, W. K. 1992b. Prey-handling and envenomation strategies of prairie rattlesnakes (Crotalus v. viridis) feeding on mice and sparrows. J. Herpetol. 26:496-499.

Hayes, W. K. 2007. The snake venom-metering controversy: levels of analysis, assumptions, and evidence. In press *in* W. K. Hayes, K. R. Beaman, M. D. Cardwell, and S. P. Bush (eds.), The Biology of Rattlesnakes. Loma Linda University Press, Loma Linda, California.

Hayes, W. K., P. A. Lavin-Murcio, and K. V. Kardong. 1995. Northern Pacific rattlesnakes (*Crotalus viridis oreganus*) meter venom when feeding on prey of different sizes. Copeia 1995:337-343.

Hayes, W. K., S. S. Herbert, G. C. Rehling, and J. Gennaro. 2002. Factors that influence venom expenditure in viperids and other snake species during

predatory and defensive contexts. Pp. 207-233 *in* G.W. Schuett, M. Höggren, M. E. Douglas, and H.W. Greene (Eds.), Biology of the Vipers. Eagle Mountain Publ., Eagle Mountain, Utah.

Herbert, S. S. 1998. Factors influencing venom expenditure during defensive bites by cottonmouths (*Agkistrodon piscivorus*) and rattlesnakes (*Crotalus viridis*, *Crotalus atrox*). M.S. Thesis, Loma Linda University, Loma Linda, California.

Hodgson, W. C., and J. C. Wickramaratna. 2006. Snake venoms and their toxins: Australian perspective. Toxicon. 48:931-40.

Hostettler, S., and W. Nentwig. 2006. Olfactory information saves venom during prey-capture of the hunting spider Cupiennius salei (Araneae: Ctenidae). Funct. Ecol. 20:369-375.

Jackson, K. 2003. The evolution of venom-delivery systems in snakes. Zool. J. Linn. Soc. 137: 337-354.

Kardong, K. V. 1982. Comparative study of changes in prey capture behavior of the cottonmouth (*Agkistrodon piscivorus*) and Egyptian cobra (*Naja haje*). Copeia 1982:337-343.

Kardong, K. V. 1986a. The strike behavior of the rattlesnake, *Crotalus viridis oreganus*. J. Comp. Psychol. 100:304-314.

Kardong, K. V. 1986b. The predatory strike of the rattlesnake: when things go amiss. Copeia 1986:816-820.

Kardong, K. V., and V. L. Bels. 1998. Rattlesnake strike behavior: kinematics. J. Exptl. Biol. 201:837-850.

Kardong, K. V., and P. A. Lavin-Murcio. 1993. Venom delivery of snakes as high-pressure and low-pressure systems. Copeia 1993:644-650.

Kardong, K. V., and T. L. Smith. 2002. Proximate factors involved in rattlesnake predatory behavior: a review. Pp. 253-266 *in* G. W. Schuett, M. Höggren, M. E. Douglas, and H. W. Greene (eds.), Biology of the Vipers. Eagle Mountain Publ., Eagle Mountain, Utah.

Kardong, K. V., T. Kiene, and V. Bels. 1997a. Evolution of trophic systems in squamates. Neth. J. Zool. 47:1-17.

Klauber, L. M. 1972. Rattlesnakes: Their Habits, Life Histories and Influence on Mankind, 2 Vols. University of California Press, Berkeley.

Kochva, E. 1960. A quantitative study of venom secretion by *Vipera palaestinae*. Am. J. Trop. Med. Hyg. 9:381-390.

Kochva, E. 1987. The origin of snakes and evolution of the venom apparatus. Toxicon 25:65-106.

Kochva, E. 1978. Oral glands of the reptilia. Pp. 43-161 *in* C. Gans and K. A. Gans (eds.), Biology of the Reptilia. Vol. 8. Academic Press, New York.

Kuhn, B. F., M. J. Rochelle, and K. V. Kardong. 1991. Effects of rattlesnake (*Crotalus viridis oreganus*) envenomation upon the mobility and death rate of laboratory mice (*Mus musculus*) and wild mice (*Peromyscus maniculatus*). Bull. Md. Herpetol. Soc. 27:189-194.

Lavin-Murcio, P., B. G. Robinson, and K. V. Kardong. 1993. Cues involved in relocation of struck prey by rattlesnakes, *Crotalus viridis oreganus*. Herpetologica 49:463-469.

Leinz, E. F., and T. R. F. Janeiro-Cinquini.1994. Influence of the extraction interval on the venom yield from snakes *Bothrops jararaca*. The Snake 26:67-70

Mackessy, S. 1991. Morphology and ultrastructure of the venom glands of the northern Pacific rattlesnake, *Crotalus viridis oreganus*. J. Morphol. 208:109-128.

McCue, M. D. 2006. Cost of producing venom in three North American pitviper species. Copeia 2006:818-825.

McCue, M. D. 2007. Prey envenomation does not improve digestive performance in western diamondback rattlesnakes (*Crotalus atrox*). J. Exp. Zoo. DOI: 10.1002/jez.411.

McCue, M.D. In press. Cost of producing venom in North American pitvipers. Copeia. Orzack, S., and E. Sober (Eds.). 2001. Adaptationism and Optimality. Cambridge Univ. Press, Cambridge.

Minton, S. A. 1987. Poisonous snakes and snakebite in the U.S.: a brief review. Northwest Sci. 61:130-137.

O'Connell, D. J., and D. R. Formanowicz, Jr. 1998. Differential handling of dangerous and non-dangerous prey by naive and experienced Texas spotted whiptail lizards, *Cnemidophorus gularis*. J. Herpetol. 32:75-79.

Owings, D. H., and R. G. Coss. 2007. Hunting California ground squirrels: constraints and opportunities for northern Pacific rattlesnakes. In press *in*

W. K. Hayes, K. R. Beaman, M. D. Cardwell, and S. P. Bush (eds.), The Biology of Rattlesnakes. Loma Linda University Press, Loma Linda, California.

Radcliffe, C. W., D. Chiszar, and B. O'Connell. 1980. Effects of prey size on poststrike behavior in rattlesnakes (*Crotalus durissus, C. enyo* and *C. viridis*). Bull. Psychon. Soc. 16:449-450.

Rodriguez-Robles, J. A., and R. Thomas. 1992. Venom function in the Puerto Rican racer, *Alsophis portoricensis* (Serpentes: Colubridae). Copeia 1992: 62-68.

Rosenberg, H. 1967. Histology, histochemistry and emptying mechanisms of the venom gland of some elapid snakes. J. Morph. 122:133-156.

Rowe, M. P., and D. H. Owings. 1990. Probing, assessment, and management during interactions between ground squirrels and rattlesnakes. Part 1: risks related to rattlesnake size and body temperature. Ethology 86:237-249.

Schaeffer, R. C., Jr., S. Bernick, T. H. Rosenquist, and F. E. Russell. 1972. The histochemistry of the venom glands of the rattlesnake *Crotalus viridis helleri*: II. Monoamine oxidase, acid and alkaline phosphatase. Toxicon 10:295-297.

Shine, R., L. Sun, M. Fitzgerald, and M. Kearney. 2002. Antipredator responses of free-ranging pit vipers (Gloydius shedaoensis, Viperidae). Copeia 2002:843-850.

Spiller, H. and G. Bosse. 2003. Prospective study of morbidity associated with snakebite envenomation. J. Tox. Clin. Tox. 41:125-130.

Stephens, D. W., and J. R. Krebs. 1986. Foraging Theory. Princeton Univ. Press., Princeton, New Jersey.

Stewart, J., and W. F. Gilly. 2005. Piscivorous behavior of a temperate cone snail, Conus californicus. Biol. Bull. 209:146-153.

Thomas, R. G., and F. H. Pough. 1979. The effect of rattlesnake venom on digestion of prey. Toxicon 17:221-228.

Thorpe, R. S., W. Wuster and A. Malhorta (eds.). 1997. Venomous Snakes: Ecology, Evolution and Snakebite. Clarendon Press, Oxford, 1997.

Tu, A. T. (ed.) 1977. Venoms: Chemistry and Molecular Biology. John Wiley and Sons, New York.

Tu, A. T (ed.). 1982. Rattlesnake Venoms: Their Actions and Treatment. Marcel Dekker, New York.

Tu, A. T. (ed.) 1991. Handbook of Natural Toxins. Vol. 5. Reptile Venoms and Toxins. Marcel Dekker, New York.

Underwood, G. 1967. A contribution to the classification of snakes. London: British Museum (Natural History).

Underwood, G., and E. Kochva. 1993. On the affinities of the burrowing asps *Atractaspis* (Serpentes: Atractaspididae). Zool. J. Linn. Soc. 107:3-64

Vidal, N. 2002. Colubroid systematics: evidence for an early appearance of the venom apparatus followed by extensive evolutionary tinkering. J. Toxicol. Toxin Rev. 21:21–41.

Werman, S. D. 2007. Phylogeny and the evolution of ß-neurotoxic phospholipases A_2 (PLA_2) in the venoms of rattlesnakes, *Crotalus* and *Sistrurus* (Serpentes: Viperidae). In press *in* W. K. Hayes, K. R. Beaman, M. D. Cardwell, and S. P. Bush (eds.), The Biology of Rattlesnakes. Loma Linda University Press, Loma Linda, California.

Whitaker, P. B., K. Ellis, and R. Shine. 2000. The defensive strike of the eastern brownsnake, Pseudonaja textilis (Elapidae). Funct. Ecol. 14:25-31.

Young, B. A. 2007. Perspectives on the regulation of venom expulsion in snakes. In press *in* W. K. Hayes, K. R. Beaman, M. D. Cardwell, and S. P. Bush (eds), The Biology of Rattlesnakes. Loma Linda University Press, Loma Linda, California.

Young, B. A., and K. Jackson. 2007. Functional specialization of the extrinsic venom gland musculature within the crotaline snakes (Reptilia: Serpentes) and the role of the M. Pterygoideus Glandulae. In press *in* W. K. Hayes, K. R. Beaman, M. D. Cardwell, and S. P. Bush (eds.), The Biology of Rattlesnakes. Loma Linda University Press, Loma Linda, California.

Young, B. A., and K. V. Kardong. 2007. Mechanical and physiological influences on venom expulsion in the western diamondback rattlesnake, *Crotalus atrox*. Journal of Experimental Zoology 307A:18-27.

Young, B. A., and K. Zahn. 2001. Venom flow in rattlesnakes: mechanics and venom metering. J. Exptl. Biol. 204:4345-4351.

Young, B. A., M. Blair, K. Zahn, and J. Marvin. 2001a. Mechanics of venom expulsion in *Crotalus*, with special reference to the role of the fang sheath. Anat. Rec. 264:415-426.

Young, B. A., C. E. Lee, and K. M. Daley. 2002. Do snakes meter venom? Bioscience 52:1121-1126.

Young, B. A., M. Phelan, J. Jaggers, and N. Nejman. 2001b. Kinematic modulation of the strike of the western diamondback rattlesnakes (*Crotalus atrox*). Hamadryad 26:316-349.

Young, B. A., M. Phelan, M. Morain, M. Ommundsen, and R. Kurt. 2003. Venom injection in rattlesnakes (*Crotalus*): peripheral resistance and the pressure-balance hypothesis. Can. J. Zool. 81:313-320.

Young, B. A., K. Zahn, M. Blair, and J. Lalor. 2000. Functional subdivision of the venom gland musculature and the regulation of venom expulsion in snakes. J. Morphol. 246:249-259.